UNIVERSITY OF EDINBURGH.

STUDIES ON FUCOIDIN

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CONTENTS.

INTRODUCTION.

Any summary of work done on the polysaccharides of marine algae must necessarily be somewhat disjointed and incomplete, for, though a considerable amount of research on these compounds has been carried out in the last decade, the chemistry of seaweed polysaccharides is still obscure. Comparatively few seaweeds have been examined, and in even fewer cases has the complete structure of the polysaccharides concerned been worked out.

The polysaccharides of seaweed include gel-forming materials such as agar and carragheen mucilage (called geloses by Tseng (1)), and non-gelling materials such as alginic acid (a poly-uronide) and laminarin (a polyglucoside with 1:3 linkages). Marine algae have been divided into four main groups (2), depending on the pigment which predominates.

(a) Chlorophyceae or green seaweeds.

(b) Phaeophyceae or brown seaweeds.

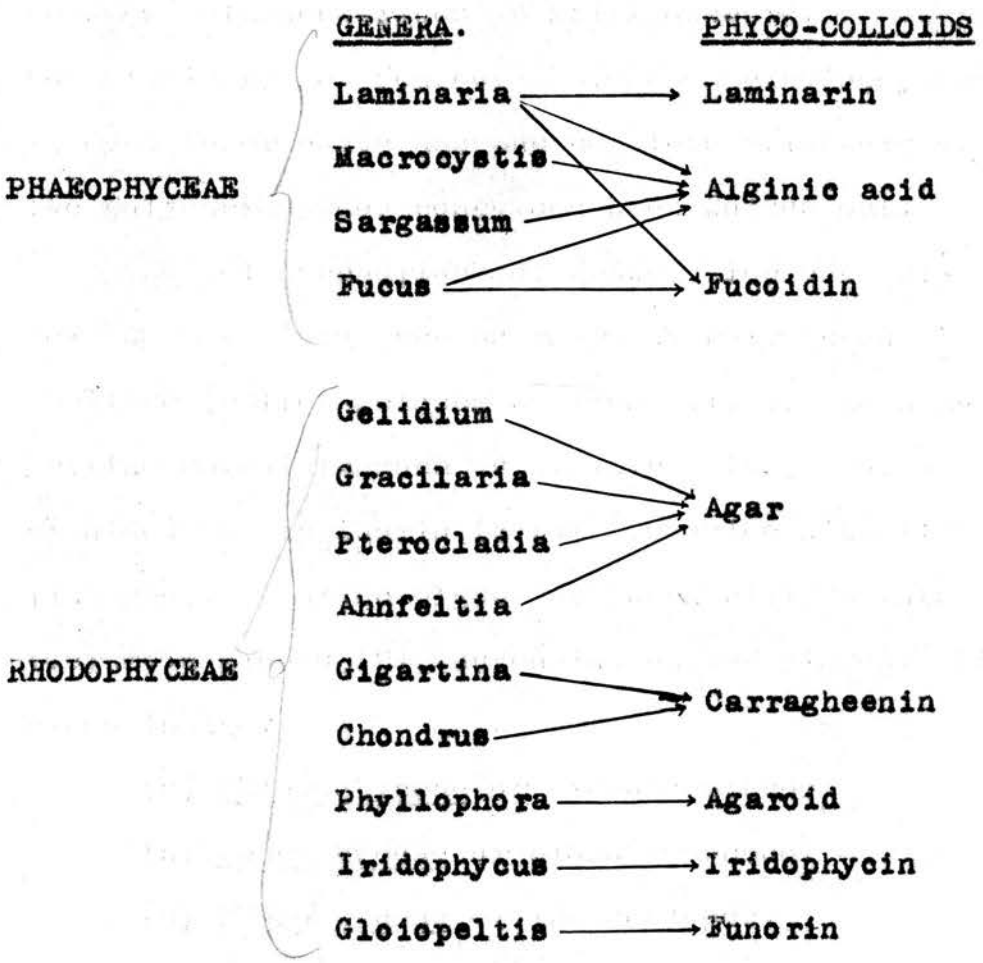
(c) Rhodophyceae or red seaweeds.

(d) Cyanophyceae or blue-green seaweeds.

Although the red and brown seaweeds have received some study, very little is known of the polysaccharides of the green and blue-green seaweeds.

Tseng/

Tseng has proposed the name of phyco-colloids for the polysaccharides derived from the brown and red seaweeds and capable of forming colloidal systems when dispersed in water. The diagram below, abbreviated from Tseng's table, gives his tentative arrangement of seaweed polysaccharides.



For the purposes of this introduction it will be convenient to divide the polysaccharides to be considered into two groups - carbohydrate sulphuric esters, and other polysaccharides (e.g. alginic acid).

A. Carbohydrate sulphuric esters.

Before discussing this group in detail it is perhaps advisable to summarise the general characteristics of these compounds.

Polysaccharide ethereal sulphates are extracted from seaweeds with water or dilute acids as salts and purified by prolonged dialysis. The simplest general formula is

$$(R.O.SO_2.OM)_n$$

$R.O.SO_2.OM$ represents the repeating unit in the polysaccharide.

R is the monosaccharide repeating unit.

M is the metal - Na, K, $\frac{Ca}{2}$ or $\frac{Mg}{2}$.

n is unknown in most cases.

Generally the formula is not quite as simple as the above, for R is often a mixture of two or more carbohydrate residues. While in such compounds the metal is ionised, e.g. in a salt the calcium can be precipitated quantitatively with ammonium oxalate, the suplhate is not ionised, and an aqueous solution of an ethereal sulphate will give no precipitate with barium chloride until after hydrolysis with hydrochloric acid.

On ignition, an ethereal sulphate gives an ash, consisting of M_2SO_4, and it is evident that during ignition half the sulphate is lost as SO_3

$$2\ R.O.SO_2.OM \longrightarrow M_2SO_4 + SO_3 + \text{products of combustion.}$$

Since/

Since on hydrolysis with acids an ethereal sulphate gives the free sugar, or sugars, together with sulphuric acid

$$R.O.SO_2.OM + HCl + H_2O \longrightarrow R.OH + H_2SO_4 + MCl$$

the sulphate content of the ash will only be half that contained in the hydrolysis mixture. This 1:2 ratio of sulphate in the ash to sulphate after hydrolysis is characteristic of an ethereal sulphate.

It has been found in practice, however, that this 1:2 ratio does not always hold, the sulphate in the ash being often very much less than half the sulphate after hydrolysis. This difference may be due either to the fact that the ethereal sulphate cannot be represented by such a simple formula, or to the loss of sulphur from the ash, the large amount of carbon present reducing the sulphate present to sulphite or even sulphide. Hence, during ignition, sulphur may be lost as sulphur dioxide, while, in dissolving the ash in hydrochloric acid, sulphur may again be lost from M_2S as H_2S. This difficulty has been overcome by converting the ash to sulphate with sulphuric acid before estimating sulphate. Nelson and Cretcher (3) and Lunde, Heen and Öy (4) have referred to this low sulphate in the ash unless the ash is determined as sulphate.

The actual metal found in these ethereal sulphates/

sulphates appears to have little bearing on the characteristics of the polysaccharide. The same ethereal sulphate can be obtained as the calcium salt from one alga and an alkali metal salt from another. Two different salts of the same ethereal sulphate may be obtained from the same alga, if the two samples of alga are collected at different parts of the world, or if the method of extraction is different. The alkali metal salt may, for example, be soluble in cold water, while the calcium salt may be insoluble, in which case the cold extract will contain chiefly the alkali metal salts, and the hot extract will be chiefly the calcium salt.

(1) Fucoidin.

This water-soluble ethereal sulphate occurs along with the water-insoluble polyuronide, alginic acid, in various common brown seaweeds. It was first isolated and named by Kylin (5), who extracted it from various species of Laminaria and Fucus. In addition to sulphate, he claimed that both pentoses and methyl-pentoses were present.

Bird and Haas (6) isolated fucoidin from various Laminaria species by washing the freshly gathered weed in distilled water and precipitating the exudate by the addition of absolute alcohol. After purification by dialysis and reprecipitation, the polysaccharide/

polysaccharide yielded 30-33% ash, chiefly calcium sulphate. Sulphate in the ash (15.1%) was found to be half the total sulphate (30.3%), which, together with the fact that the calcium was ionised and could be precipitated by ammonium oxalate, suggested that fucoidin was the calcium salt of an ethereal sulphate. As regards the carbohydrate portion, Bird and Haas confirmed the presence of a methylpentose first claimed by Kylin, and, in addition, found evidence for the existence of 7.3% of a uronic acid (which may, however, have come from alginic acid).

Lunde, Heen and Öy (4), recognising that the purification of fucoidin was a matter of extreme difficulty, attempted to obtain as pure and unchanged a sample as possible by collecting the viscous drops exuded from Laminaria digitata on short standing, and purifying it by boiling with alcohol. It is difficult to understand how samples collected in this way do not contain a proportion of salts of alginic acid, which are present in considerable quantities in this species. Estimation of the ash gave a 26-30% yield, while the sulphate estimation on the ash (17-19%) was approximately half that of the sulphate estimated in the hydrolysis products (35.5-37.7%). The estimation of sulphate in the ash, however, did not agree with the assumption that the substance was exclusively/

exclusively a calcium salt, for, in theory, calcium sulphate contains 70.34% sulphate, while estimations on the ash gave 63-66%. Analysis of the ash showed it consisted chiefly of sodium sulphate (28.43% Na in ash) with some potassium (3.48%), calcium (3.44%) and small quantities of magnesium sulphate.

An attempt was made by the same workers to estimate quantitatively the fucose in the hydrolysis product by distillation with 13% hydrochloric acid according to Tollens (7), and formation of the resulting methylfurfural as the phlorogluoide. It was thus estimated that 33-37% of the polysaccharide was fucose. Bird and Haas found in fucoidin 7.3% of a uronic acid, calculated by estimating the carbon dioxide given off on hydrochloric acid distillation according to Tollens. Thus, in the estimation of fucose, Lunde et al. should have found furfural phlorogluoide in the phlorogluoide precipitate. But furfural phlorogluoide is, in contrast to methylfurfural phlorogluoide, only very slightly soluble in alcohol, and Lunde's phlorogluoide precipitate was completely soluble in alcohol. It was therefore assumed that the uronic acid in the preparation of Bird and Haas was due to an impurity. The absence of furfural also excluded the presence of pentoses which were present according to Kylin, but the possible/

possible presence of hexoses could not be excluded, since according to Kullgren and Tydén (6) they give 1-2% ω-hydroxymethylfurfuraldehyde, the phlorogluçide of which is soluble in alcohol.

The sum of the components of fucoidin which have been estimated, i.e. metals, sulphate and fucose, makes up about 80% of the substance, leaving 20% to be accounted for. Lunde proposed the formula

$$(R - R' - O.SO_2 - OM)_n$$

R is fucose; R' is another carbohydrate complex; M is Na, K, $\frac{Ca}{2}$ or $\frac{Mg}{2}$; n is unknown.

(2) Polysaccharide from Macrocystis pyrifera.

This water-soluble polysaccharide, which appears to be similar to fucoidin, was isolated by Hoagland and Lieb (9). The polysaccharide was precipitated from the aqueous extract, and repeatedly precipitated from acid solution to remove salts. The final product contained approximately 35% calcium sulphate. The phenylosazone of fucose was obtained from the hydrolysis liquid. No other sugar was found. An ethereal sulphate grouping was shown to be present by Nelson and Cretcher (3), who put forward the probable formula $(R - O - SO_2 - OH)_4$ for this ester where R is a methylpentose, probably exclusively fucose. They also found a uronic acid which was believed to come from alginic acid.

(3) Chondrus crispus polysaccharide.

Most of the early investigations on Chondrus crispus were made using the actual seaweed rather than the aqueous extract. As a consequence of this, the results obtained are extremely complex and often discordant. Flückiger and Obermayer (10) found that treatment with nitric acid gave mucic acid, while Bente (11) isolated laevulic acid on heating the seaweed with mineral acid. The isolation of galactose was first reported by Haedicke, Bauer and Tollens (12), and the presence of a small proportion of pentose or methylpentose was indicated by the work of Müther and Tollens (13). Fructose was reported by Lintner, Düll and Kiermayer (14), who considered this was proved present by the formation of hydroxymethylfurfuralphenylhydrazone. Carragheen was stated by Sebor (15) to be a complex carbohydrate containing galactose, glucose and fructose residues, with a small amount of pentose.

An important step in the elucidation of the problem was made when Haas and Hill (16) in 1921 reported the presence of two polysaccharide ethereal sulphates in Irish moss. Haas (17) later found a method of separating these two polysaccharides. One fraction was readily soluble in cold water, while the other was only soluble in hot water. Both fractions had/

had high ash contents not reducible on prolonged dialysis, and the hot extract was shown to be chiefly a calcium polysaccharide ethereal sulphate.

Further work on the nature of both fractions was carried out by Russell-Wells (18), who, after proving that the cold extract was also an ethereal sulphate, showed that the hot extract always contained more calcium and less sodium and potassium than the cold extract. Haas and Russell-Wells (19) confirmed the presence of fructose and glucose in the hot extract. The sulphate groups were found to be extremely difficult to remove with alkali, only 20% removal being obtained in 16 hours with 3% sodium hydroxide at 110°.

In Canada, M.R. Butler (20) found that the inorganic constituents of the polysaccharide of Irish moss collected there, varied considerably from those obtained by Bird and Haas. Instead of the 2:1 ratio of sulphate after hydrolysis to sulphate in the ash, proposed by the Haas ethereal sulphate formula, she obtained a 3:1 ratio and, moreover, found a high potassium content and low calcium content. By dialysing against appropriate salts she obtained pure potassium and calcium salts for which the 2:1 ratio held. The 3:1 ratio was explained by the fact that/

that the extract was a mixture of potassium, calcium and ammonium ethereal salts.

Further work on the Chondrus polysaccharide was carried out by Dillon and O'Colla (21), who attempted acetolysis with acetic anhydride and sulphuryl chloride. They obtained two sulphate-free acetates, composed exclusively of galactose units. Considerable degradation had taken place however.

Considerable progress in the study of the structure of the polysaccharide was made by means of methylation experiments carried out by Buchanan, Percival (E.E.) and Percival (E.G.V.) (22). They found that apart from the mineral constituents, the cold and hot extracts were essentially identical. In both extracts galactose was found to be the main constituent (34% in the cold extract, and 37% in the hot extract), as shown by hydrolysis with acid and estimation of the sugar as galactosemethylphenyl-hydrazone. From the galactose-free portion of the hot extract both glucosazone and β-methylglucoside tetra-acetate were obtained, but in such small yields that it was obvious that glucose was not the main constituent of this portion. In the case of the cold extract, on the other hand, no glucose was found, but it was estimated from colorimetric determinations that about 20-22% of a ketose was present.

Neither/

Neither extract could be acetylated, so direct methylation with dimethyl sulphate and caustic potash according to Bell (23) was used. Even after repeated methylations, the methoxyl content of the extracts never reached that required for a dimethyl hexose ethereal sulphate. The methylated product still retained the ethereal sulphate group, and it was assumed that little or no degradation had taken place.

From both the methylated cold extract (OMe 14.5 %) and hot extract (OMe 14.2 %), 2:6-dimethyl and 2-methyl galactose as syrups, were obtained on hydrolysis. The isolation of 2:6-dimethyl galactose gave considerable insight into the mode of linkage and the location of the ethereal sulphate group in the repeating galactose units. Since positions 2 and 6 were occupied by methoxyl groups in the dimethyl galactose, these positions were regarded as being free in the polysaccharide. Moreover, the units were assumed to be pyranose in character, since the rate of hydrolysis of both extracts with acid was not in agreement with the presence of galactofuranose units.

The position of the ethereal sulphate group raises the problem of the relative stabilities of sulphuric ester groups on different carbon atoms, and

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a brief summary of work in this field might profitably be attempted at this stage.

In order to determine the position of the sulphate residues in agar and other polygalactose sulphates, the examination of D-galactose sulphates was begun by Percival and co-workers (24,25), who prepared a barium D-galactose 6-sulphate. While acid hydrolysis did not provide a means of distinguishing between related hexose sulphates, alkaline hydrolysis did show differences, and by the action of saturated barium hydroxide on the methyl-glycosides of D-glucose and D-galactose sulphates, two methylanhydrohexosides were produced. Moreover, it was shown that the barium salts of the sulphates of the methylglycosides of D-galactose, D-glucose and D-mannose all yielded the corresponding methyl 3:6-anhydrohexosides together with unchanged methyl-hexosides. The formation of anhydro rings is of value in locating sulphate groups, which, in the above cases, must be on C_3 or (more likely) C_6.

In more recent work, Percival (26) has shown that on alkaline hydrolysis 1:2-isopropylidene-D-glucofuranose 6-sulphate gives 1:2-isopropylidene-3:6-anhydro-D-glucofuranose and 1:2-isopropylidene-D-glucofuranose, while 1:2-isopropylidene-D-glucofuranose 3-sulphate (which is very slowly hydrolysed) gives/

gives only 1:2-isopropylidene-D-glucofuranose. From these results, and from the examination of other compounds with sulphate groups on C_3 or C_6, the important conclusion was drawn that with sulphates (unlike p-toluenesulphonates) ethylene oxide rings are not necessarily formed on treatment with alkali, but 3:6 anhydro rings are formed.

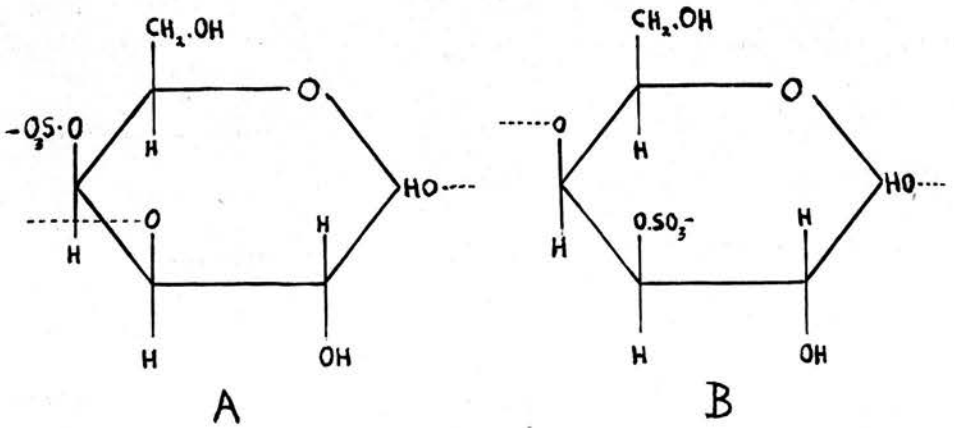
Later work by Percival and Duff (28), however, showed that when conditions were suitable and the formation of a 3:6 anhydro ring was prevented by substitution, the hydrolysis of an ethereal sulphate readily gave rise to the production of an ethylene oxide ring, as in the case of the p-toluenesulphonates. The previous conclusion that 5:6 anhydrides were not produced (26), based on the failure to obtain L-idose derivatives from barium 1:2-monoacetone glucofuranose 6-sulphate was based on an unsound foundation since Reichstein et al. (29) showed that 1:2-monoacetone 5:6-anhydroglucofuranose was converted to the corresponding 3:6 anhydride on treatment with alkali, and not into L-idose derivatives, contrary to previous reports (30). For this reason, the experiments were repeated with the hydroxyl group on C_3 blocked by a methoxy group, which had the additional advantage of making the barium ethereal sulphate under investigation soluble in methanol, thus making possible a smooth/

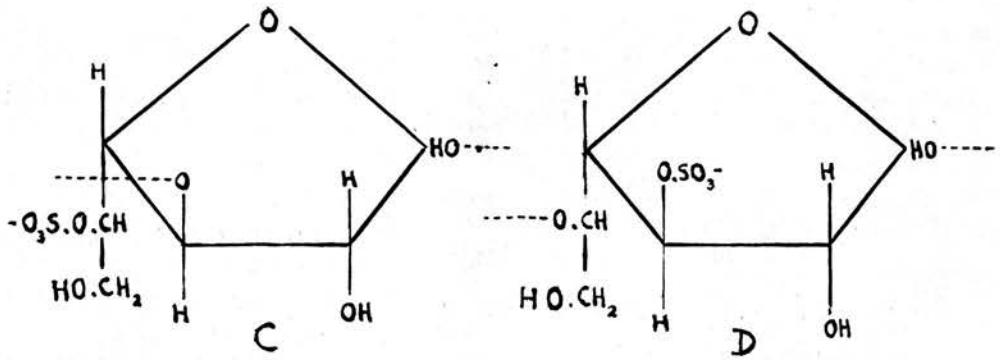
smooth reaction with sodium methoxide. Percival and Duff found that barium 1:2-monoacetone 3-methyl glucofuranose 6-sulphate yielded the corresponding 5:6 anhydride on treatment with sodium methoxide.

On the other hand, neither barium 4:6-benzylidene α-methylglucoside sulphate nor barium 6-methyl β-methylgalactopyranoside 2-sulphate gave anhydrides on treatment in methanol with sodium methoxide.

Considering the Chondrus polysaccharide once more, it was assumed that the ethereal sulphate was probably located on C_4, because the rate of removal of sulphate by 4% sodium hydroxide was extremely slow, taking 73 hours to remove 80%. It has been shown in a number of cases (27,24) that where hydrolysis of hexose sulphates or toluenesulphonates is difficult there is no possibility of interaction with another hydroxyl group to form an anhydro ring.

If we assume that the sulphate groups are directly attached to the galactose residues, four possible formulae must be considered.





It has been shown (28) that barium 3-methyl 1:2-monoacetone glucofuranose 6-sulphate is readily transformed into the corresponding 5:6 anhydride by sodium methoxide. It is highly probable, therefore, that the sulphate groups in B, C and D would be readily eliminated by alkali, with formation of ethylene oxide rings in the first instance. In addition, B would form a 3:6 anhydride by analogy with the methylglucofuranoside 3-sulphates (26). The most likely arrangement is, therefore, A in which the galactopyranose residues are linked through the 1 and 3 positions. The above arguments, based on the resistance to hydrolysis with alkali of the sulphate groups, apply only if these residues are directly attached to the galactose units. It is possible, however, that the sulphate groups are attached to those building units of the molecule which have not, as yet, been identified, with these units interposing between the sulphate groups and the triply linked/

linked galactose residues.

Since the rotations of the hot and cold extracts and their methylated derivatives were positive, and on account of the preponderance of D-galactose residues, the glycosidic linkage was assumed to be α-.

The problem of the constitution of the non-galactose portion of the polysaccharide was investigated by Young and Rice (31), who claimed to have isolated 2-ketogluconic acid in considerable quantity. After hydrolysis with oxalic acid and potassium oxalate in an atmosphere of nitrogen, and evaporation to dryness, the residue was extracted with alcohol, in which galactose is almost completely insoluble. The alcohol-soluble portion was treated with acetone and copper sulphate, and gave a crystalline derivative, identified as diacetone 2-ketogluconic acid.

On methylation of the polysaccharide, Young and Rice claimed to obtain a crystalline product - m.p. 130-140° (decomp.); $[\alpha]_D^{18°}$ +48.0°; OMe 15.2% ash 18.2% Hydrolysis, followed by complete methylation and distillation, gave a fraction boiling at 140-165°/0.05mm. (OMe 56.4%). This on treatment with methanolic ammonia gave a crystalline amide, claimed to be 2:3:4:6-tetramethyl 2-ketogluconic acid amide. If this result is correct, then it has some bearing/

bearing on the claims of earlier workers to have isolated fructose from this polysaccharide.

(4) Polysaccharide from Gigartina stellata.

Gigartina stellata is a seaweed very similar to Chondrus crispus and is, in fact, collected with that species and marketed with it under the name of Carragheen or Irish moss. The polysaccharide obtained by hot water extraction from Gigartina stellata has been investigated by Percival and Dewar (32), and has been found to be similar to the Chondrus crispus polysaccharides already discussed.

The polysaccharide from the hot extract was an ethereal sulphate $[\alpha]_D^{15^\circ}+51^\circ$; ash 17.5 % (as sulphate), giving calcium 3.7 % magnesium 1.0 % sulphate 12.7 % calculated on the weight of the hot extract, the total sulphate being 23.8 %. Hydrolysis of the hot extract with $\frac{N}{2}$ oxalic acid, followed by neutralisation with barium carbonate, gave D-galactose (40 %), together with the barium salt of an acid (30 %), the constitution of which has not been decided.

As with the Chondrus crispus polysaccharides, direct methylation of the hot extract was slow, but it could be acetylated readily in the cold after preliminary treatment with pyridine. Simultaneous deacetylation and methylation yielded a partly methylated/

methylated product, the methoxyl content of which was raised by several similar treatments to a value of 18-20%. The methylated polysaccharide so obtained closely resembled the original hot extract:- $[\alpha]_D^{15°}$ +43° in water; ash 18.2% (as sulphate), giving calcium 3.8%; magnesium 0.9%; sulphate 12.8% (calculated on weight of methylated hot extract); and total sulphate 24.7%.

Hydrolysis of the methylated polysaccharide and suitable treatment, gave as the main product a dimethyl methylgalactoside, from which on methylation, hydrolysis, and treatment with aniline a good yield of tetramethyl D-galactopyranose anilide was obtained, thus indicating the absence of substitution on C_5. The dimethyl methylgalactoside ditoluene p-sulphonate underwent no reaction with sodium iodide in acetone (33), indicating the presence of a methoxyl group on C_6. Removal of the glycosidic methoxyl gave a crystalline dimethyl galactose, which on osazone formation, gave pure 6-methyl galactosazone, proving substitution on C_2 and C_6. The sugar was also characterised by the preparation of the acid, lactone, amide, phenylhydrazide, β-methylgalactoside, 3:4-monoacetone β-methylgalactoside and anilide. The properties of these compounds were in good agreement with those recorded by Bell (34) in his studies on

synthetic/

synthetic 2:6-dimethyl galactose. Thus this dimethyl galactose isolated in crystalline form from a natural source for the first time was 2:6-dimethyl β-D-galactopyranose.

As in the case of Chondrus crispus, the rate of removal of sulphate from the hot extract with normal sodium hydroxide at 100° was exceedingly slow (62% in 3 days), indicating that the sulphate group was located on C_4, and so the chief building unit of the polysaccharide was assumed to be an anhydro-galacto-pyranose 4-sulphate, which was joined to adjacent units by an α-1:3 linkage as in the case of the Chondrus crispus polysaccharides. The absence of α glycol groups was confirmed by the failure of the polysaccharide to react with periodic acid.

(5) Polysaccharide from Dilsea edulis.

Barry and Dillon (35) in 1945 isolated from Dilsea edulis (Stackhouse) a polysaccharide which, on hydrolysis with dilute hydrochloric acid, gave free sulphuric acid and galactose. No other sugars could be detected, and the compound was therefore a galactan sulphuric ester, of which the active principle of the mucilage was a salt. Estimation of sulphuric acid residues gave figures lying midway between the values for one sulphate group on every four/

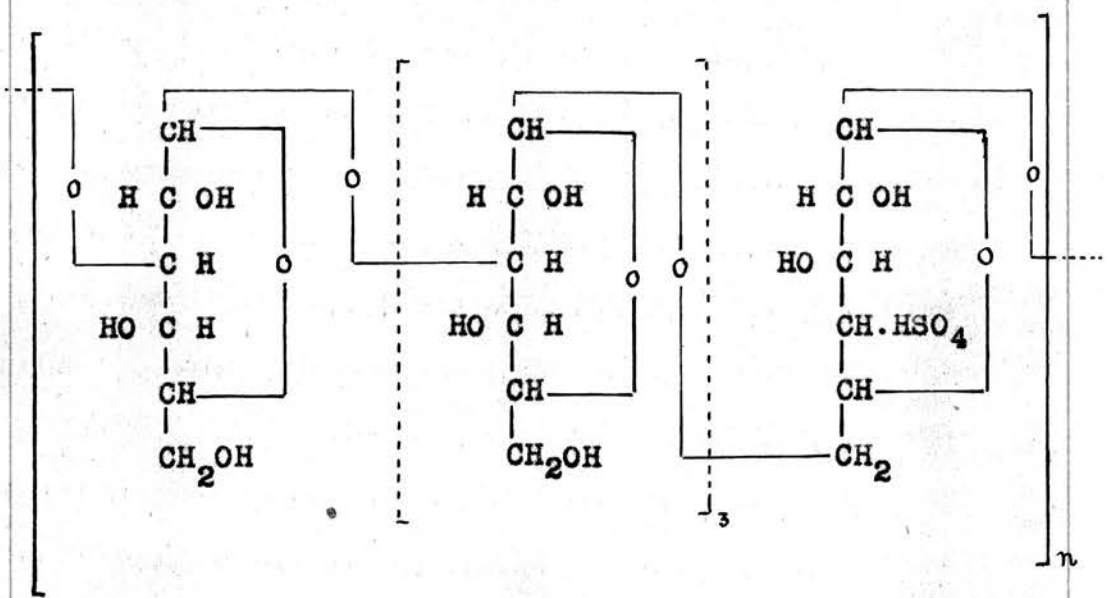
four and one on every five units.

The behaviour of the galactan ester towards periodic acid threw some light on its constitution. When the galactan ester was kept with a dilute periodic acid solution, the specific rotatory power of the solution fell from +50° to +15° in about six hours, indicating a considerable change in the molecule. No further decrease in rotation took place on several days' standing. The oxidised product, on hydrolysis by acid or takadiastase, which hydrolyses it slowly, gave glyoxalosazone and galactosazone in good yield. It also yielded mucic acid on the usual treatment. It was clear, therefore, that some galactose units were oxidised while others were not. The combined sulphuric acid was not removed by the periodic acid.

The oxidised galactan sulphuric ester, on treatment with phenylhydrazine acetate, gave an immediate precipitate in the cold of glyoxalosazone (36). From the percentage weight of this osazone it appeared that about one galactose unit in every five was oxidised. After removal of the glyoxal-osazone, excess alcohol precipitated a white flocculent substance, which dried to a yellowish-white powder. This, on treatment with periodic acid, followed/

followed as above by the phenylhydrazine reagent, gave a further fraction of glyoxalosazone, showing that the original treatment with phenylhydrazine of the oxidised galactan ester had brought about a fragmentation of the molecule, and thus exposed further galactose units to the action of the periodic acid.

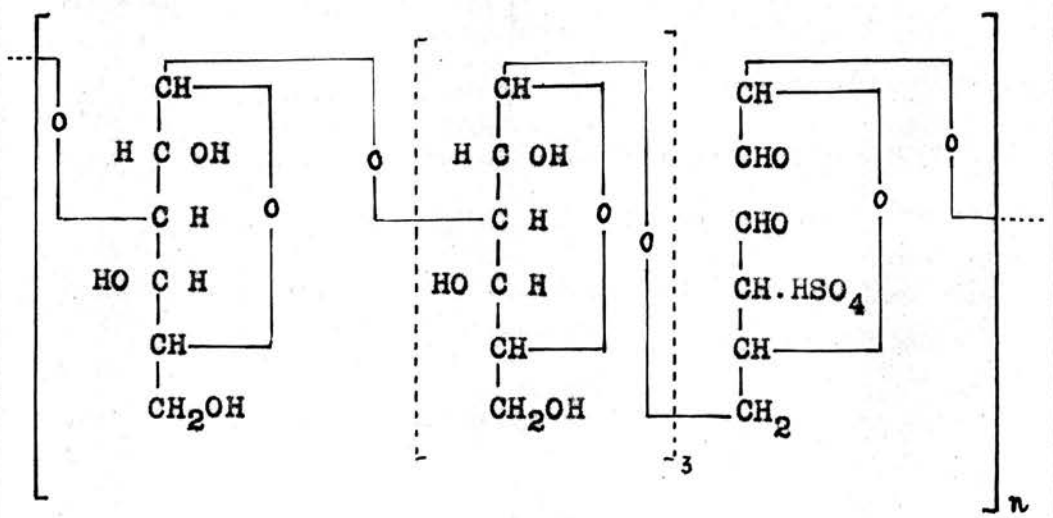
As a result of these experiments, Barry and Dillon proposed the following formula for the polysaccharide:-



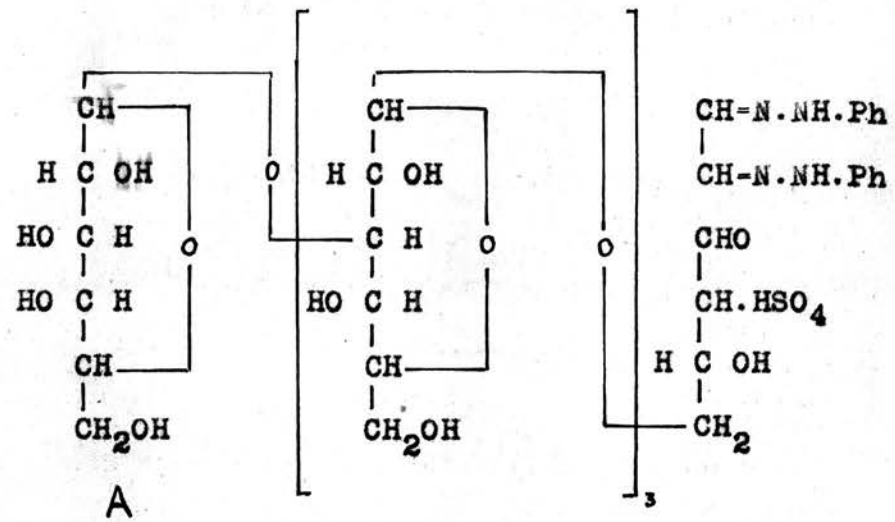
The repeating unit of the molecule is regarded as being composed of a linear chain of five galacto-pyranose units, the first four of which are linked 1:3, while/

while the linkage between the fourth and fifth is 1:6. The fifth galactose unit is also esterified with sulphuric acid at the fourth carbon atom. This fifth unit, which is the one open to attack by periodic acid, could alternatively have been represented with the sulphuric acid on C_6, and the linkage through C_4. Percival and his co-workers (24,25) had, however, already shown that methyl-galactoside 6-sulphate on alkaline hydrolysis yielded 3:6-anhydromethylgalactoside, whereas Barry and Dillon's product showed considerable resistance to alkaline hydrolysis, retaining 50% of combined sulphate after boiling with normal sodium hydroxide for 13 hours.

The evidence thus favoured placing the linkage of this unit at C_6, and the sulphuric ester group on C_4. Such a compound would be attacked by periodic acid at the fifth unit, and would give the compound:-



Phenylhydrazine acting on this compound gives glyoxalosazone, and is presumed to cause fragmentation of the molecule to the following compound, the unit A of which is now open to attack by periodic acid:-



(6) Polysaccharide from Irideae laminarioides.

Hassid (37) extracted from the red alga Irideae laminarioides a polysaccharide ethereal sulphate, iridophycin, which appeared on examination to be a simple sodium galactan ethereal sulphate. Examination of the hot extract gave $[\alpha]_D^{+}69.2^{c}$; ash 25.4% sulphate in the ash 17.5% total sulphate 34.5 - 37.2% Thus the 1:2 ratio of sulphate in ash to/

to sulphate after hydrolysis proves this polysaccharide to have an ethereal sulphate group. The ash was shown to be chiefly sodium sulphate with small amounts of calcium and magnesium sulphates.

An interesting development in the study of this polysaccharide was the preparation of the free galactan sulphuric acid by electrodialysis. The amorphous powder obtained had a pH of 2.86 for a 1% solution, and an equivalent of 366, the theoretical equivalent of a pure galactan sulphuric acid $C_6H_9O_4.O.SO_2.OH$ being 242. This high value was attributable to part of the sulphate being removed during dialysis. The original sodium salt gave a titration curve typical of a salt of a strong acid, indicating that the free galactan sulphuric acid was a strong acid.

On acid hydrolysis of the polysaccharide no other sugar but galactose could be isolated. Acetylation with pyridine and acetic anhydride yielded a diacetate which still retained the ethereal sulphate. Methylation by the method of Haworth and Learner (38), followed by two treatments with Purdie's reagents, yielded the dimethyl sodium galactan sulphate (OMe 20.0% $[\alpha]_D$ +17.2° in chloroform). This on hydrolysis gave a reducing, sulphate-free syrup, which did not crystallise. On forming the glycoside and distilling,

a/

a crystalline dimethyl methylgalactoside was obtained, the constitution of which, however, was not determined.

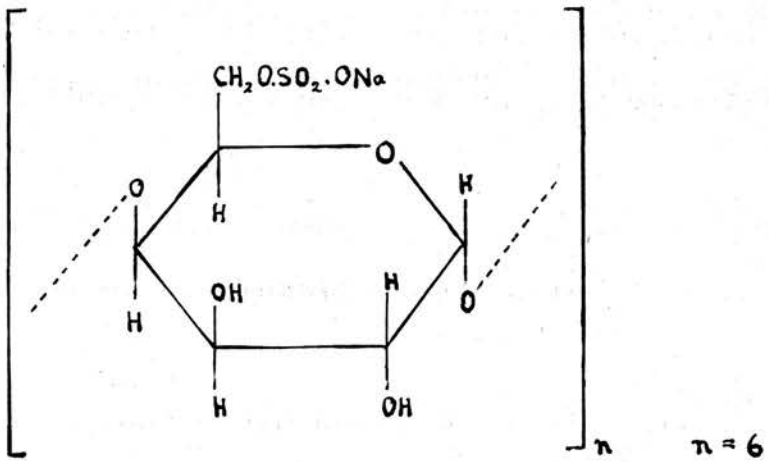
Hassid found that the sodium ethereal sulphate grouping could be removed, either with 0.5 N sulphuric acid or 5 % baryta, to yeild the pure galactan, without destroying the carbohydrate complex. If this is the case, it seems peculiar that methylation with dimethyl sulphate and caustic soda did not also remove the sulphate group. On methylating this sulphate-free galactan, a crystalline trimethyl galactan (OMe 44.5 %) was obtained, which on hydrolysis gave a syrupy trimethyl galactose. This on oxidation with bromine water and then nitric acid, followed by esterification, gave a syrup which Hassid claimed was a dimethyl arabodimethoxyglutarate.

As a result of these experiments, Hassid rejected the idea of a 1:6 linkage, for he claimed that the fact that a dimethoxy compound was obtained proved that C_6 was occupied by a methoxyl group in the trimethyl galactan. Assuming a pyranose ring structure, a trimethoxyglutaric acid would have been obtained had C_6 been free.

Since no osazone could be prepared from the trimethyl galactose obtained on hydrolysis as described above, it appeared that C_2 carried a methoxyl group, and so the 1:2 linkage was also rejected./

rejected. The rejection of the 1:5 linkage was based on the assumption that the pyranose form of galactose was present, and that of the 1:3 linkage on the fact that at that time (1935) such a linkage had not been discovered in a naturally occurring substance, while the 1:4 linkage was common. Since then, of course, the 1:3 linkage and not the 1:4 linkage has been shown to be common in polysaccharides containing galactose, e.g. agar (39), damson gum (40), gum arabic (41) and the carragheen polysaccharides (22).

The ethereal sulphate group was deduced to be on C_6 for steric reasons and, by determining the molecular weight of the sodium galactan sulphate by the micro-method of Rieche (42), Hassid estimated the chain length to be about six sodium galactose sulphate units.



Since Hassid's investigations, however, other researches have somewhat invalidated his conclusions.

In/

In addition to the preponderance of 1:3 linkages in galactose polysaccharides, there is also the question of formation of osazones from hexoses containing a methoxyl on C_2. This has been shown to be quite common, e.g. the formation of 4:6-dimethyl galactosazone from 2:4:6-trimethyl galactose (39), and 6-methyl galactosazone from 2:6-dimethyl galactose (22,43). The fact that an osazone cannot be prepared from a partially methylated sugar cannot be taken as evidence that C_2 is occupied by a methoxyl group.

(7) Agar.

Agar, an extremely important polysaccharide, is extracted from Gelidium corneum and other species of Gelidium and closely related algae. The property of agar of forming gelatinous solutions is widely utilised in the preparation of culture media, as a component of emulsions, in foodstuffs, adhesives etc.

In addition to D-galactose, the hydrolysis products include the enantiomorphous L-galactose and sulphuric acid. Agar apparently exists as the calcium or magnesium salt of the sulphuric acid ester of a polysaccharide consisting of D- and L-galactose units (44).

Percival and Somerville (39) were the first to establish the fundamental features of the structure of/

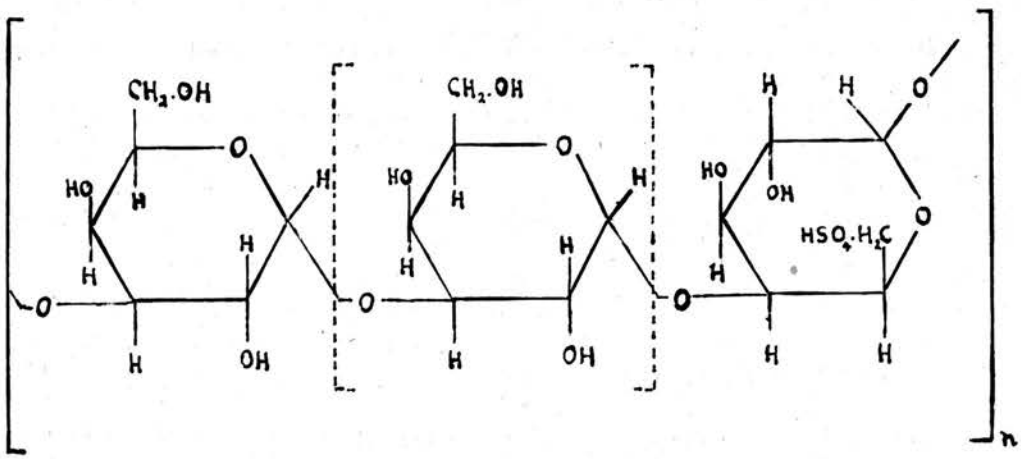
of this important polysaccharide. Using the methylation procedure, they showed that the major part of the agar molecule consisted of D-galactose residues united by 1:3 glycosidic linkages, chiefly of the β-type. The sulphuric acid group esterified with galactose appeared to be hydrolysed during acetylation or methylation, since no sulphate could be detected in agar acetate or methylated agar.

It was later established that L-galactose was an integral part of this polysaccharide, by the isolation of a derivative of L-galactose from the hydrolysis products of methylated agar (45), confirming Pirie's observation of this sugar (46) from the acetolysis products of agar. Further confirmation of this point was made by Araki (47), who reported the isolation of a disaccharide from the hydrolysis products of methylated agar. This was pentamethyl-D-galactoside-3:6-anhydro-methyl-L-galactoside, the D- and L-galactose derivatives being united probably through C_1 and C_4. It was further shown that the methylated L-galactose derivative contained a 3:6 anhydro ring. But, inasmuch as Percival and Duff (25) demonstrated that alkaline hydrolysis of methylgalactoside 6-sulphate led to the formation of 3:6 anhydromethylgalactoside, it was assumed that the 3:6 anhydro form of L-galactose obtained/

obtained from agar was not an original constituent, but a residue formed in the course of hydrolysis of L-galactose 6-sulphuric ester in agar.

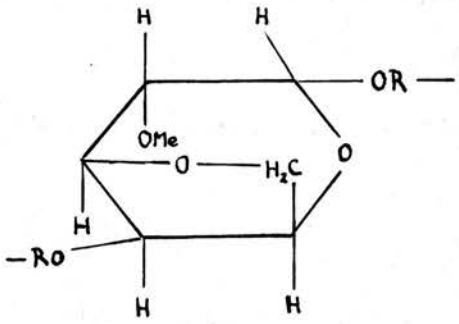
W.G.M. Jones and Peat (48) detected among the hydrolysis products of methylated agar 2:5-dimethyl 3:6-anhydro-L-galactonic acid. A carboxylic acid had not hitherto been found in agar and the L-galactonic acid was considered to be an artifact produced from a 3:6-anhydro-L-galactose residue by atmospheric oxidation during the preparation.

According to these authors, agar is the sulphuric acid ester of a linear polygalactose, in which the repeating unit is composed of nine D-galactopyranose residues terminated at the reducing end by one residue of L-galactose. The constitution is thus represented as follows:-

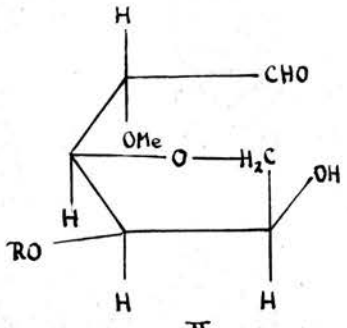


In the course of methylation the sulphuric acid residue is presumed to be lost and a 3:6 anhydro ring/

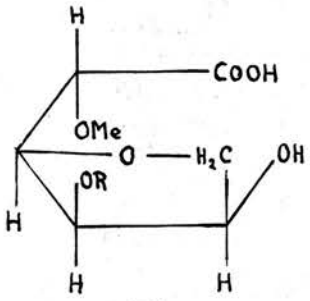
ring (I) appears in the L-galactose member. It has been shown that anhydroglycosides of this type are exceedingly sensitive to acid, and if acid conditions supervened during the preparation of methylated agar, the aldehydo form (II) would result, and would readily pass by atmospheric oxidation into the acid (III), thus explaining the isolation of 2:5-dimethyl 3:6-anhydro-L-galactonic acid.



I



II



III

The fact is stressed that, whereas the D-galactose residues of agar are mutually combined by 1:3 linkages, the L-galactose residue is attached to the chain through C_4 and it may be significant in regard/

regard to the biological synthesis of this polysaccharide that D-galactose 1-sulphate substituted at C_3 is theoretically convertible by a simple intramolecular oxidation and reduction change into L-galactose 6-sulphate substituted at C_4. It is claimed that D-galactose 1-sulphate plays a similar part in the symthesis of agar to that played by glucose 1-phosphate in the synthesis of starch (49).

Percival and Thomson (50), however, are of the opinion that the above structure is over-simplified. Their results regarding the relative proportions of hydrolysis products in methylated agar are somewhat at variance with those of Jones and Peat. Moreover they point out that the methoxyl content for a model of methylated agar on the lines suggested above should be 42% whereas the highest recorded value is not greater than 35% for a representative sample.

Furthermore, the formulation of agar suggested by Jones and Peat, which required a sulphur content of 1.8% was not supported by the analyses of agar isolated from Gelidium latifolium (51) which contained 0.36% sulphur, or from Gracilaria confervoides (0.43% sulphur) and Gelidium crinale (0.47% sulphur) (52). It was clear that none of the above specimens contained sufficient sulphate to account for the proportion of 2:4-dimethyl 3:6-anhydro-/

anhydro-β-methyl-L-galactoside (9% (48); 11.5% (50)) isolated from methylated agar, on the former of which the "sulphate formula" proposed by Jones and Peat was based.

It was also suggested by Percival (52) that, since there is no direct evidence that the sulphate groups remaining in the agar after isolation are located on C_6 of the L-galactose residues, the ethereal sulphate could be situated on C_3, since experiments have shown that methylglucofuranoside 3-sulphates yield 3:6-anhydrides on alkaline hydrolysis. Moreover, if the hydroxyl groups on C_3 were esterified, the agar would possess no α-glycol groupings and would therefore not react with periodic acid, thus providing an alternative explanation for the results of Barry and Dillon (51).

Mention might be made at this stage of a polysaccharide obtained from Gelidium amansii by Miyake and Hayasi (53). Alkaline extraction gave a polysaccharide, free of uronic groups, from which D-galactose, L-arabinose, and a methylpentose, probably fucose, were isolated.

B. Other important seaweed polysaccharides.

(1) Alginic acid.

Dilute alkali extracts from brown seaweeds (Phaeophyceae) a polysaccharide called algin or alginic acid, which has some industrial interest because of its property of forming viscous mucilaginous solutions, and because of its possible use as a textile fibre. The polysaccharide may be obtained by extracting the seaweed with alcohol to remove mannitol and pigments, then with cold dilute hydrochloric acid to remove the soluble sulphuric esters, and finally with cold 2% sodium carbonate in which the alginic acid swells to give a thick mucilaginous extract, from which on acidification the free acid may be obtained.

Two of the earliest workers in this field, Hoagland and Lieb (9), claimed to find xylose on hydrolysis of a sample, and for a time it was believed, due to the work of Atsuki and Tomoda (54), that the acidic nucleus, to which the pentose units were attached, was D-glucuronic acid which was claimed to be isolated as the cinchonine salt by Schmidt and Vocke (55). Doubt as to the presence of xylose in the polysaccharide arose when Cretcher and Nelson (56) reported that their preparations of alginic acid from various species contained as much as/

as 98% uronic acid. They suggested that any pentose found in the hydrolysis products was due to decarboxylation of a uronic acid unit.

Alginic acid on titration gave a neutralisation equivalent of from 176 to 184, indicating that the carboxyl groups were free. Since decarboxylation of the free uronic acids took place very readily, it was not at all easy to find suitable conditions for hydrolysis of the polysaccharide. Eventually Nelson and Cretcher (57) obtained 80% hydrolysis by standing the polysaccharide with 80% sulphuric acid at room temperature for five days. In this case, the cinchonine salt obtained was neither that of glucuronic or galacturonic acid. Oxidation of the barium salt with bromine, and formation of the diamide and diphenylhydrazide of the dibasic acid, gave derivatives of D-mannosaccharic acid, and so the conclusion was reached that alginic acid was a pure polyuronide of D-mannuronic acid, an acid which had not previously been known to exist in natural products. Independent investigations by Bird and Haas (6) and Miwa (58) confirmed the presence of D-mannuronic acid. Later, improved methods of preparation gave the crystalline α- and β-D-mannuronic acids, and identification was completed by comparison of mannuronic acid lactone with

synthetic/

synthetic D-mannuronic acid lactone obtained by reduction of D-mannosaccharic acid dilactone (59).

Complete methylation of the polysaccharide with dimethyl sulphate could not be achieved but diazomethane was more satisfactory. Experiments were carried out by Hirst, Jones (J.K.N.) and Jones (W.O.) (60), on a degraded alginic acid obtained by boiling a sample with 10 % hydrogen chloride in methanol. On methylation and hydrolysis, the methyl ester of 2:3-dimethyl-D-mannuronide was obtained, which on further hydrolysis yielded 2:3-dimethyl-D-mannuronic acid. This, on oxidation with bromine, formed 2:3-dimethyl-D-mannosaccharic acid and more extensive oxidation with periodic acid gave glyoxylic acid and the semi-aldehyde of meso-dimethoxysuccinic acid, identified as the crystalline dimethyl ester of meso-dimethoxysuccinic acid. The linkage was thus either 1:4 or 1:5, since the methoxyl groups were on C_2 and C_3. The extreme stability of the polysaccharide to acid hydrolysis favoured the 1:4 linkage, while the β-configuration for the linkages seemed probable because of the high laevorotation of the polysaccharide.

Confirmatory evidence was produced by the direct oxidation of alginic acid with periodic acid followed by/

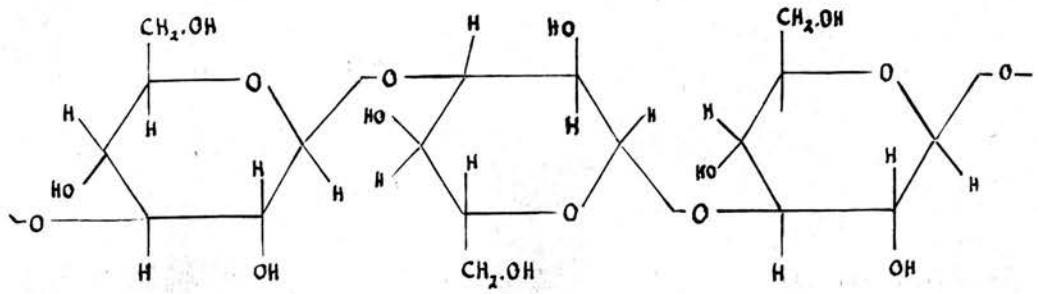
by bromine, carried out by Lucas and Stewart (61). Each mannuronic unit with this reagent would be expected to yield a 2:3 dialdehyde which would be converted by bromine to a tricarboxylic acid. The dialdehyde on acid hydrolysis would yield glyoxal and D-erythuronic acid, while the bromine oxidation product would yield glyoxylic and mesotartaric acids. From the former 42% of glyoxal was obtained experimentally, and from the latter 25% of meso-tartaric acid, indicating that C_2 and C_3 were not involved either in ring or bridge linkage.

Important confirmation of the type of linkage in alginic acid was made by the X-ray studies of Astbury (62) and Palmer and Hartzog (63). They found the period along the fibre axis to be 8.7 Å, in contrast to 10.3 Å for cellulose. This is due to the fact that when hexopyranose rings are constructed in the "chair" form using standard bonds and angles, the formation of β-linkages between C_1 and C_4 in contiguous rings is possible in two ways. In the first case the rings are inclined at an angle of 20° with one another, and the resultant chain is almost linear. This represents cellulose and agrees with the observed figure of 10.3 Å. In the second case where the rings are at an angle of 90°, the theoretical value would be 8.7 Å, which agrees with that/

that found for alginic acid. Thus it appears that the structure of alginic acid is more buckled than that of cellulose.

(2) Laminarin

The constitution of this polysaccharide which occurs in the Laminaria species was studied by Barry (64), who found it to be a polyglucose. Methylation and hydrolysis of the polysaccharide gave 2:4:6-trimethylglucopyranose, indicating that the linkages between the units were 1:3 linkages, while the negative rotation of laminarin suggested they were of the β-type. Though at first it was believed that laminarin was an ethereal sulphate, it was soon realised that under the proper conditions of extraction laminarin contained negligible amounts of combined sulphate. Hydrolysis of laminarin with oxalic acid, or an enzyme from snail juice, produced a new disaccharide for which Barry (65) proposed the name laminaribiose. Its probable structure is glucose-3-β-glucoside. The arrangement of the glucose residues in laminarin may be represented thus:-



The extent of oxidation of laminarin with periodic acid was used by Barry (66,67) as an "end-group assay" for this polysaccharide. This determination was based on the fact that periodic acid reacts with the group ═C(OH)-C(OH)═, splitting the carbon bond and oxidising each group to an aldehyde. In a polysaccharide such as starch or cellulose the linkage is 1:4, and so the necessary pair of adjacent C-OH groups is provided by C_3 and C_2. If, however, as in the case of laminarin, the linkage is 1:3, there is no pair of adjacent C-OH groups available, and the polysaccharide cannot be attacked by periodic acid, except at terminal nonaldehydic glucose units which can supply the requisite groups.

It was found in practice that a small but definite reduction of periodic acid took place when laminarin was oxidised. Subsequent oxidation with bromine yielded a polysaccharide whose end group contained/

contained two carboxyl groups. Estimation of the dicarboxylated end group indicated a chain length of 16 glucose units for laminarin. This chain length did not agree however with that obtained by the Haworth-Hirst (68) method of end-group assay, which indicated chain length of 74 glucose units.

Laminarin and a yeast polyglucose are of special interest in that they are the only polysaccharides known in which the glucose residues are joined by 1:3 glycosidic linkages.

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P A R T I.

Investigations on Methods of Extraction and Purification, and Studies on the Properties of the Polysaccharide.

EXPERIMENTAL.

Selection of a suitable species of seaweed.

In an endeavour to find a species of seaweed reasonably rich in fucoidin, various specimens were examined. Since fucoidin is a carbohydrate sulphuric ester, it was considered that a suitable method of estimating the amount present would be to hydrolyse the seaweed with hydrochloric acid, and estimate the sulphate content.

Accordingly, weighed amounts (2-3 g.) of various species of dried ground seaweed, kindly supplied by the Scottish Seaweed Research Association, were hydrolysed by boiling for five hours with 80 ml. of 5 % hydrochloric acid. The residue was filtered off, washed with hot water, and the sulphate precipitated by adding slowly 10 ml. of boiling barium chloride (3 %) as described in Cumming and Kay's "Quantitative Chemical Analysis" (1). After allowing to stand overnight, the precipitate was filtered through a sintered glass crucible (1 G 4), dried at 150° C, and weighed.

Since, according to Lunde (2), the amount of fucoidin is at its highest in August - September, and at its lowest in April when fresh fronds are forming/

forming, April and August samples were taken.

Summary of results.

Species.	% Sulphate. APRIL.	% Sulphate. AUGUST.
L. digitata	2.99	3.24
L. Cloustoni	3.3	3.59
L. saccharina	1.65	2.07
A. nodosum	5.8	6.08
F. vesiculosus	7.25	5.52
Saccharriza bulbosa	-	3.39

These experiments did not point to an especially good source of fucoidin.

PRELIMINARY EXPERIMENTS.

Some whole fronds of the April samples of L. digitata, F. vesiculosus and A. nodosum were soaked in distilled water for several days. At intervals the extracts were examined.

(a) On adding barium chloride solution to portions of the extracts, a flocculent white precipitate was obtained in the case of L. digitata, while with the other two extracts only faint precipitates were obtained.

(b) With mercuric chloride only a faint precipitate was obtained in each case.

Since/

Since only faint reactions were obtained, it was decided to concentrate the extracts under reduced pressure at 50°. The various concentrated extracts were poured into alcohol (500 ml.). It was found on testing the precipitates that all contained a considerable amount of chloride, so a portion was kept for a sulphate estimation, and the rest was dissolved in water and dialysed till free from chloride, when it was again concentrated under reduced pressure and poured into alcohol.

	% Sulphate before dialysis.	% Sulphate after dialysis.
L. digitata	4.42	6.8
F. vesiculosus	8.76	10.64
A. nodosum	7.42	9.1

Experiments with a fresh frond of L. digitata.

A fresh frond of L. digitata (October sample) was obtained, and various attempts were made to separate the slimy film which covered it. The frond was soaked in water and allowed to drip into a large beaker. Later, it was covered with water and agitated for some time, the blisters which formed on the frond being cut open to assist extraction. After 48 hours the combined extracts (2 l.) were/

were poured off, concentrated to about 30 ml., and poured into alcohol, when a pale brown precipitate was obtained. This was filtered off and dried *in vacuo* at 50°. Yield 18 g. The frond was also dried and weighed. (Weight 70 g.).

15 g. of the precipitate was dissolved in 100 ml. water and dialysed till free from chloride (72 hrs.). After concentration and precipitation, a portion was dried *in vacuo* and sulphate estimations, ash, and sulphate determinations on the ash, were carried out. Yield 11 g.

	Sulphate.	Ash	Sulphate on Ash
Undialysed ppt. (A)	7.84%	10.7%	84.2%
Dialysed ppt. (B)	10.72%	12.8%	73.8%

From the figures obtained it was obvious that the polysaccharide contained considerable amounts of impurities, so various methods of purification were next considered.

EXPERIMENTS ON THE PURIFICATION OF THE POLYSACCHARIDE.

(A) Purification by fractional precipitation.

A quantity of the polysaccharide B (5 g.) was dissolved in water (45 ml.). To the solution, fixed volumes of alcohol were added with stirring, and the resulting/

resulting precipitates were centrifuged and dried overnight *in vacuo* at 50°, when sulphate estimations were carried out. After excess alcohol had been added, the filtrate was evaporated to dryness *in vacuo*.

Since the quantities of precipitates obtained were comparatively small, a micro-method of determining sulphate had to be used.

Micro-determination of sulphate (3).

The compound, sufficient to give about 10 mg. barium sulphate was weighed in a small tube, transferred to a small beaker, and 5 ml. water added. To this was added 5 ml. of 18% hydrochloric acid and the compound was hydrolysed by boiling for 5 hours, (as in the case of *Chondrus crispus* (16)). About 20 mg. of barium chloride was weighed out, dissolved in 10 ml. of water and two drops of dilute hydrochloric acid were added. This solution was brought to the boiling point and added drop by drop, with constant stirring, to the boiling solution of the hydrolysed polysaccharide (which sometimes had to be filtered, the residue being carefully washed with hot water). After allowing to settle overnight, the precipitate was transferred to the filter-stick, prepared as described in Pregl's "Quantitative Organic Micro-Analysis"/

Analysis" (3), washed with warm water and then acetone, and dried at 150° in a heating-block.

Summary of Results.

5.08 g. of polysaccharide B was dissolved in 45 ml. water.

Fraction	Volume of alcohol added	Weight of precipitate	% Sulphate
I	20 ml.	0.28 g.	14.15
II	20 ml.	0.41 g.	16.83
III	25 ml.	0.43 g.	17.66
IV	50 ml.	0.92 g.	18.23
V	excess	2.87 g.	20.78
VI	(residue after evaporation of filtrate)	0.0545 g.	10.41

While the fractions showed a steady increase in sulphate content and thus presumably an increase in the purity of the polysaccharide, the results obtained were not sufficiently satisfactory to cause this method to be considered further.

(B) Attempted purification using basic lead acetate.

Basic lead acetate has been reported to precipitate fucoidin (4), and so attempts were made to obtain pure fucoidin by this method in order to avoid the long process of concentrating the extracts.

A small portion of the undialysed precipitate A (pg. 49)/

(pg. 49) was dissolved in water, and basic lead acetate solution was added, whereupon a white precipitate formed. This was centrifuged, suspended in water, and hydrogen sulphide was passed through the suspension for 20-30 mins. Air was then bubbled through, but the lead sulphide did not coagulate. Gentle heating was also unsuccessful, and only partial coagulation was obtained on adding a little dilute sulphuric acid. On neutralising with barium carbonate and filtering through a pad of barium carbonate, a clear solution was obtained, but this yielded only a faint precipitate on concentrating and pouring into alcohol.

On repeating the experiment with a more concentrated solution of fucoidin (0.35 g. in 20 ml.), a white solid was eventually obtained. Yield 0.082 g. Sulphate 15.8%

Since it had been observed during precipitation of the lead as lead sulphide, that the lead sulphide merely formed on the surface of the complex, the experiment was repeated with 5.7 g. of polysaccharide, but after saturation with hydrogen sulphide the suspension was shaken overnight. It was found in the morning that all smell of hydrogen sulphide had disappeared, so the solution was re-saturated and again shaken. After filtering, the filtrate was dialysed/

dialysed and when neutral was evaporated to dryness in vacuo. Yield 1.4 g.

Rotation $[\alpha]_{D}^{15°}$ -66° Sulphate 20.9%

Since it appeared probable that some of the polysaccharide was adhering to the lead sulphide precipitate, this was extracted with hot water, and, after filtering through charcoal, the solution was evaporated to dryness. Yield 3 g. Sulphate 10.47%.

As it proved extremely difficult to remove the last traces of lead, due to the formation of colloidal lead sulphide, another method of elimination had to be tried.

(C) Attempts with basic lead acetate and dilute sulphuric acid.

Some dried ground L. Cloustoni 70g. was boiled with 500 ml. water for 6 hours and the extract then treated with basic lead acetate as before. The resulting precipitate was washed with warm water (600 ml.) several times, suspended in about 150-200 ml. of water, and dilute sulphuric acid (25 ml.) was added till the solution was acid to Congo Red. The suspension was placed in a stoppered bottle and shaken for 48 hours. The precipitate became lighter in colour and after shaking, the supernatant liquid was observed to have darkened somewhat. The precipitate

was/

was centrifuged and washed twice with hot water, the washings being added to the centrifugate which was dialysed till free from acid, and evaporated to dryness *in vacuo*. The solution was then dissolved in the minimum of water and poured into alcohol. The resulting solid (C) was dried by distillation with alcohol and benzene and a micro-estimation of sulphate was carried out. Yield 1.5 g. Sulphate 17.75 %.

(D) *Attempts with lead acetate and barium hydroxide.*

0.9 g. of the polysaccharide C obtained as above was dissolved in water (80 ml.). To this was added a saturated solution of basic lead acetate till all precipitation had apparently ceased. The solution was heated for half an hour on the water-bath to assist coagulation, an excess of basic lead acetate being added to ensure complete precipitation. The precipitate was centrifuged, washed with warm water, and the washings added to the filtrate.

To the filtrate, which contained excess lead acetate, was added barium hydroxide solution (saturated in the cold) till the solution was alkaline to phenolphthalein. A fairly bulky buff precipitate was obtained. On taking a small portion of the solution/

solution and adding excess barium hydroxide, a more granular precipitate, suggestive of lead hydroxide, was obtained.

The solution was centrifuged, and to the centrifugate was added a considerable excess of barium hydroxide solution (300 ml.). After filtration, the solution was neutralised with dilute sulphuric acid, and after the resulting barium sulphate had been centrifuged off, dialysed till free from acid. It was then evaporated to dryness *in vacuo*, when a small white residue of inorganic salts was obtained.

There were thus three precipitates to be considered:-

(a) The lead acetate precipitate (presumably consisting mainly of lead alginate).

(b) The first barium hydroxide precipitate.

(c) The second barium hydroxide precipitate.

All three were washed, suspended in water, and dilute sulphuric acid added till the suspensions were acid, whereupon they were put in stoppered bottles and shaken for several hours. After being dialysed till free from acid, the suspensions were centrifuged, washed, and the centrifugates evaporated to dryness.

From (a) a small dark brown horny residue was obtained. This was dissolved in the minimum of water/

water and poured into alcohol, when a pale brown fibrous precipitate separated. After filtering and drying at 60-70°, a micro-determination of sulphate was carried out.

Found: SO_4, 30.4%.

From (b) an appreciable amount (ca. 0.1 g.) of white fibrous material was obtained. This was treated as the above sample.

$[\alpha]_D^{15°}$ -116° (c, 1.06 in water).

Found: SO_4, 31.9%.

From (c) a small amount of a white residue, shown to be inorganic salts was obtained.

A sample of polysaccharide (b) was analysed.

Found: C, 32.5; H, 5.8; SO_4, 31.9%.

(E) Attempted purification by electrophoresis (5).

In this experiment, a modified form of the electrophoresis apparatus used by Harris (5) was adopted. (Diagram overleaf).

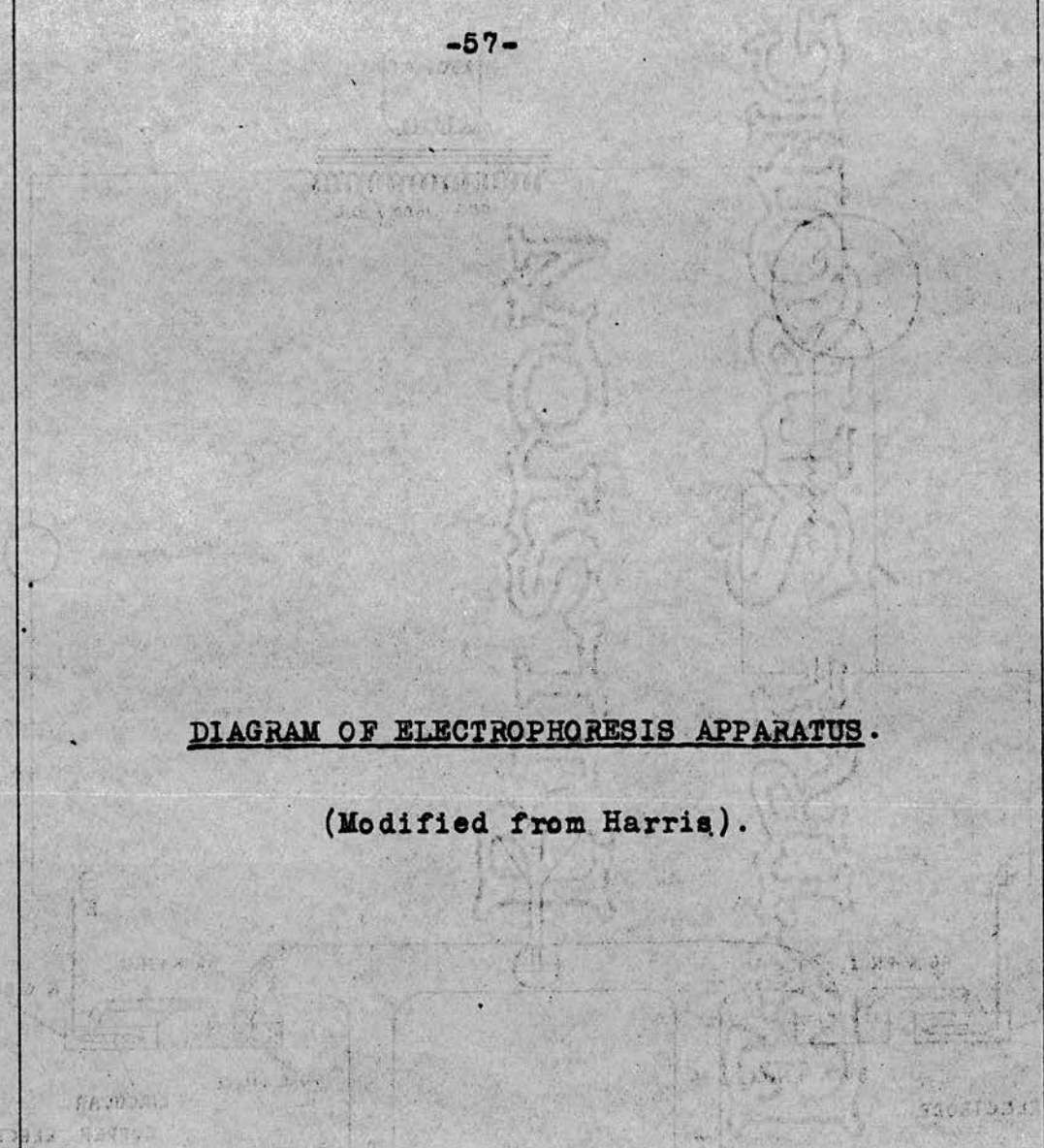
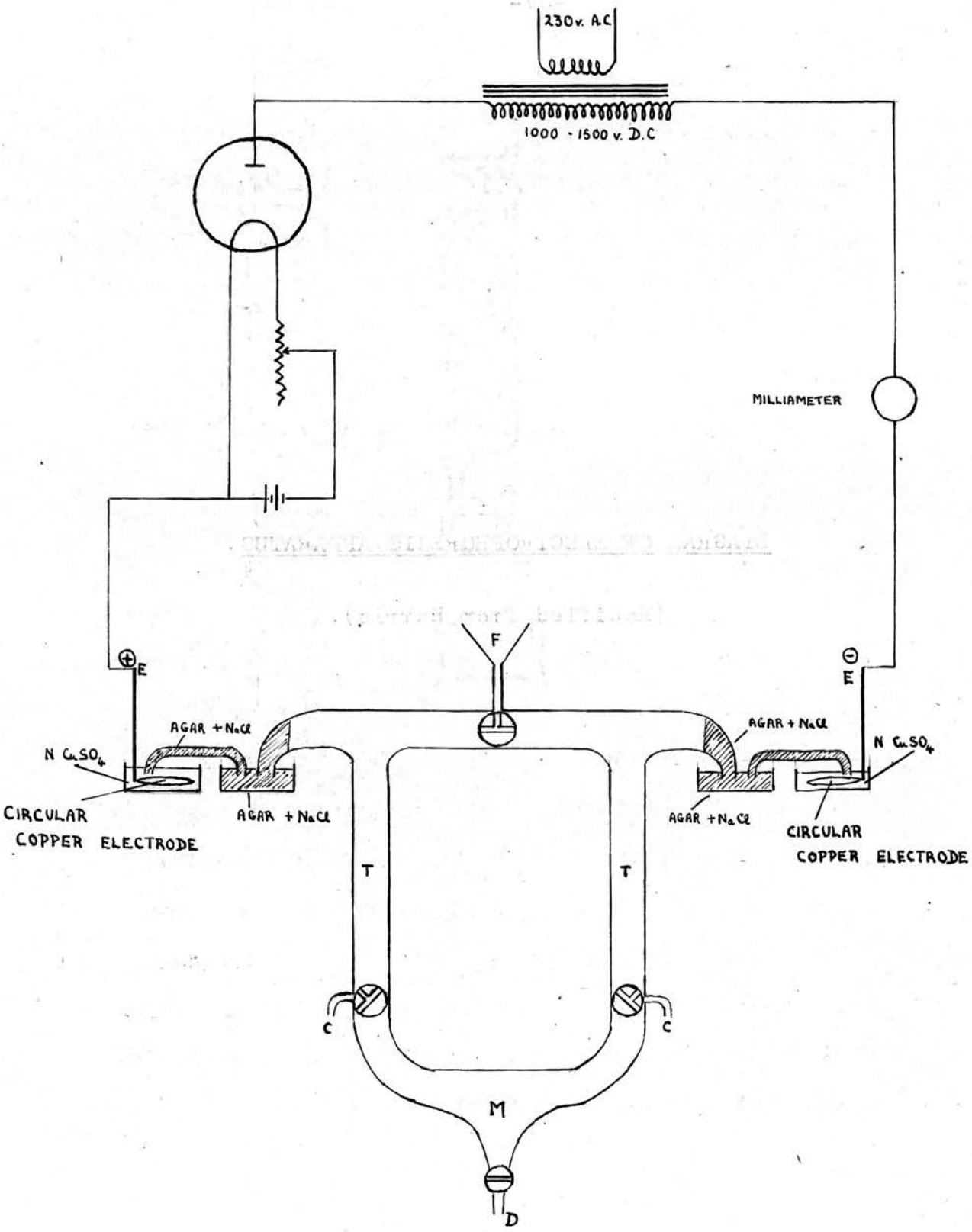


DIAGRAM OF ELECTROPHORESIS APPARATUS.

(Modified from Harris).


230v. A.C
1000 - 1500 v. D.C
MILLIAMETER
F
E
E
AGAR + NaCl
N CuSO4
CIRCULAR COPPER ELECTRODE
AGAR + NaCl
AGAR + NaCl
N CuSO4
AGAR + NaCl
CIRCULAR COPPER ELECTRODE
T
T
C
C
M
D

E are non-polarisable electrodes.

F is a safety funnel kept full of the suspending medium (in this case water) for maintaining the tubes full when in use.

C are the collecting tubes for drawing off components which have migrated into the tubes T.

T are the tubes containing the suspending medium.

M is the compartment containing the polysaccharide in the suspending medium.

D is a drain connected by rubber tubing to a filling funnel so that M can be maintained full when in use.

It was found that the apparatus in its original form had to be slightly modified. Originally Ag/AgCl electrodes were used, but it was found extremely difficult to maintain a complete circuit due to leakage of solution at the agar junction, thereby causing the agar to slip from the connecting arms. Accordingly, the arrangement shown in the diagram was adopted, an agar - sodium chloride bridge being used to maintain contact. The electrodes were flat copper discs 4 cm. in diameter.

This is the usual cataphoresis U-tube specially modified for the repeated and rapid collection of charged material during prolonged and continuous working.

In order to avoid the deleterious effect of electrolysis and heating and yet obtain a high migration velocity, it is necessary to use a low current density in conjunction with a strong directional field. For practical purposes a current density of not more than one milliamp per sq. cm. and a potential difference of 10 -50 volts per cm. are desirable. The former may be realised by using a tube of wide bore and a current limiting device, e.g. a thermionic valve with a filament considerably under-run, while the latter can only be achieved by keeping the electrolyte content of the suspending medium at a minimum and is limited by conditions such as pH. The E.M.F. necessary may be obtained from dry batteries or from the mains:-

(1) A.C. with step-up transformer and rectifying valve (as was used in this case).

(2) D.C. with a small motor generator,

each calculated to deliver 5-10 milliamps at 1500 volts.

Experimental Procedure.

The polysaccharide C (0.65 g.) was dissolved in distilled water (30 ml.). The electrophoresis was continued for two days. At the end of this time it was observed that some of the substance (coloured brown) had migrated into the positive limb, while the negative limb showed no sign of any migration having taken place. The solutions in both limbs and in the bottom tube (M) were collected and their acidity tested. None of the solutions gave any action with litmus or Congo Red.

To determine whether any of the polysaccharide had been decomposed, the solution from both limbs was tested for free sulphate with barium chloride, but nothing except a trace of barium sulphate was observed. The solution in the positive limb and that in the reservoir at the foot were then evaporated and micro-determinations of sulphate were carried out.

	Expt. I % Sulphate	Expt. II % Sulphate
Original polysaccharide C	29.6	28.7
Residue of original soln.	24.1	27.5
Fraction in positive limb	23.7	24.3

From these results it appeared that some sulphate ion had been split off in the process, and since/

since no real separation had been obtained, the method was obviously ineffective in this case.

(F) Purification using Johns-Manville "Filter-Cel".

Most samples of the polysaccharide so far obtained had been of a pale buff colour, so an attempt was made to remove all colouring matter. A quantity (ca. 2 g.) of fucoidin C obtained by the lead acetate-barium hydroxide method was dissolved in water (200-300 ml.) and shaken up with a quantity of "Filter-Cel". After standing for several days, the solution was filtered, evaporated to a small volume under reduced pressure, and precipitated by pouring into alcohol. To obtain an idea of the purity of the polysaccharide, an estimation of the fucose content, based on oxidation with periodic acid and estimation of the acetaldehyde formed, was carried out. A modification of the method of Nicolet and Shinn (6), devised by Cameron, Percival and Ross (7), was used.

Before estimations were carried out, the polysaccharide was dried overnight in vacuo at 50° C. About 20 mg. of fucoidin was hydrolysed for 5 hours with 3 ml. of 2½% oxalic acid. After neutralisation with sodium bicarbonate (and addition of excess) the estimation was carried out. The polysaccharide was/

was then dissolved in water (200 ml.) and again treated with "Filter-Cel". This treatment was repeated till the fucose content reached a constant value.

	% Fucose	% Sulphate
1st treatment	30.3	31.9
2nd treatment	32.8	31.9
3rd treatment	35.6	32.7
4th treatment	37.8	32.8

Since purification by this last method gave by far the highest figures both as regards fucose content and sulphate content, it was decided to adopt the procedure outlined above in the preparation of a considerable amount of polysaccharide.

Large-scale extraction and purification of the polysaccharide.

Dried, ground F. vesiculosus (900 g.; May, June and July 1945 samples) was heated on the steamebath with distilled water (9 l.) for 12 hours (average temperature of extract 85-90° C). The extract thus obtained was filtered through muslin, giving about 6 litres of solution. Since 4 litres was about the maximum quantity of liquid which could be dealt with at one time, this and the second extract of the same seaweed/

seaweed were divided into portions of 4 litres each.

To the solution (4 l.) was added lead acetate solution (120 g. dissolved in 100 ml. water) followed by excess solid lead acetate (160 g.). The solution, after heating on the water-bath for about one hour, was allowed to stand overnight. The very bulky precipitate of lead alginate which formed was filtered through muslin, and the resulting solution filtered through filter paper (Whatman No. 1). A saturated (in the cold) solution of barium hydroxide was then added to the filtrate till it was just alkaline to phenolphthalein. The resulting white precipitate was allowed to settle overnight and the supernatant liquid decanted. The precipitate, after filtration and washing with distilled water, was suspended in distilled water (1 litre), dilute sulphuric acid (325 ml.) was added, and the suspension shaken overnight, after which it was dialysed till free from acid (4 days). The sulphate precipitates were filtered off, the filtrate concentrated <u>in vacuo</u> to a small volume (100 ml.) and then poured into alcohol (5 l.), filtered, washed with alcohol and ether and dried. A second extraction of the seaweed was made which resulted in a further yield of extract (11 litres). This was treated as above and the

final/

final products combined. Yield 40 g.

The polysaccharide thus obtained was dissolved in water (1 litre) and shaken up with "Filter-Cel". After standing for several days, the solution was filtered, concentrated *in vacuo* and precipitated in alcohol as before. In all, four treatments with "Filter-Cel" were carried out. The final analytical figures closely resembled those found in the trial experiment. Final yield 32 g.

Found: fucose 37.7%; sulphate 32.8%.

This polysaccharide, which will in the following pages be called polysaccharide D, was used for analyses and all future experiments including the methylations described in Part II.

INORGANIC ANALYSIS OF THE POLYSACCHARIDE D.

For all quantitative work the polysaccharide was dried over phosphorus pentoxide *in vacuo* at 50-60° to constant weight. $[\alpha]_D^{15°}$ -117.8° (c, 1.5 in water).

Ash Determination.

The polysaccharide D was incinerated in a platinum crucible, and the carbon removed by ignition in a full bunsen flame to give an ash. Treatment with sulphuric acid to constant weight gave the ash as sulphate.

Weight of polysaccharide	Weight of ash	% ash as sulphate
0.10437 g.	0.02506 g.	24.0
0.09862 g.	0.02381 g.	24.15
0.09160 g.	0.02209 g.	24.11
1.0082 g.	0.2415 g.	23.96
1.0184 g.	0.2461 g.	24.18

Analysis of the ash as sulphate.

The ash as sulphate (0.2415 g.) was dissolved completely in 1:1 concentrated hydrochloric acid : water (20 ml.) and the solution made up to 100 ml. in a standard flask. Several of these standard ash solutions were prepared and used for the following determinations:-

Sulphate was determined on 25 ml. solution gravimetrically as barium sulphate.

Sodium was determined on 20 ml. as sodium zinc uranyl acetate (8).

Potassium was determined on 25 ml. as dipotassium monosodium cobaltinitrite (9).

Calcium was determined on 50 ml. as calcium oxalate by the single precipitation method (10). The calcium oxalate was then dissolved in dilute sulphuric acid and the solution titrated with standard potassium permanganate.

After/

After the removal of the calcium, magnesium was determined in the filtrate by precipitation with 8-hydroxyquinoline (11).

The results are tabulated below:-

	DETERMINATION I		DETERMINATION II	
	Ash	Polysaccharide	Ash	Polysaccharide
Calcium	24.54%	5.88%	23.95%	5.79%
Sodium	3.17%	0.76%	3.60%	0.87%
Potassium	0.75%	0.18%	0.42%	0.10%
Magnesium	1.00%	0.24%	0.91%	0.22%
Sulphate	73.24%	17.55%	72.03%	17.41%
	102.7		100.91	

% Ash as sulphate: I 23.96%; II 24.18%

Total sulphate 32.8%
(by hydrolysis with hydrochloric acid and precipitation with barium chloride).

Alkaline hydrolysis of the polysaccharide.

A weighed quantity of polysaccharide D (1.1082 g.) was heated with normal sodium hydroxide (200 ml.) at 100° in the presence of barium chloride (1.283 g.). At definite intervals, 15 ml. samples were withdrawn, water (15 ml.) and dilute acetic acid (5 ml.) added, and the solution centrifuged. The residual sulphate in 30 ml. of the solution was then determined by hydrolysing with hydrochloric acid, adding barium chloride/

chloride, and filtering off the barium sulphate on an ashless filter-paper (Whatman No. 42). The sulphate hydrolysed was obtained by subtracting the residual sulphate from the total sulphate.

Period of Hydrolysis (in hrs.)	Residual Sulphate (g. of $BaSO_4$)	Sulphate Hydrolysed (g. of $BaSO_4$)	% Hydrolysis
0	0.04851	-	-
½	0.04560	0.00291	6.0
1	0.04298	0.00553	11.4
4	0.04123	0.00728	15.0
10	0.03891	0.00960	19.8
20	0.03821	0.01030	26.1
30	0.03197	0.01654	34.1
36	0.03085	0.01766	36.4
48	0.02513	0.02338	48.2
56	0.02411	0.02440	50.3
72	0.02004	0.02847	58.7

Fermentation experiments with yeast.

A weighed quantity of fucoidin D (0.0913 g.) was refluxed on a boiling water-bath with N sulphuric acid, polarimetric readings being taken at intervals. When the rotation was constant, indicating that hydrolysis was complete, the solution was neutralised with sodium hydroxide and to the neutral solution was/

was added 0.25 g. of fresh baker's yeast. The solution was kept at 38° overnight and in the morning the rotation was again taken.

Rotations

During hydrolysis

	$[\alpha]_D^{15°}$ (c, 0.91 in H_2SO_4)
Initial rotation	-107.3° (in water)
After 1 hour	-24.6° (in H_2SO_4)
2 hours	-22.2°
3 hours	-22.2°
5 hours	-22.2°

After neutralisation.

	Hydrolysed Polysaccharide	Control (Glucose)
	$[\alpha]_D^{15°}$	$[\alpha]_D^{15°}$
Before adding yeast	-21.4°	+52.5°
After overnight treatment with yeast	-21.4°	+5.8°

Hydrolysis of the polysaccharide with 2% sulphuric acid and examination of the syrup obtained.

The polysaccharide D (5.95 g.) was dissolved in 2% sulphuric acid (150 ml.) and hydrolysed on a boiling water-bath for 5 hours. The solution was neutralised with barium carbonate, filtered, and evaporated *in vacuo* at 35° to give a syrup, together with a white residue. The syrup was extracted with water/

water, the solution filtered, and again evaporated to give a clear yellow syrup. Yield 3.8 g.

On treating portions of the syrup for barium (with dilute sulphuric acid) and for a uronic acid (naphthoresorcinol test) negative results were obtained in each case. Several estimations of fucose by the periodic acid oxidation method were carried out, the average result being 61.7 % fucose in the syrup.

In an endeavour to prove the presence or absence of glucose, a further fermentation experiment was carried out on the syrup. 0.09408 g. of the syrup was dissolved in 10 ml. of water and after the rotation had been taken, a little yeast was added and the solution kept at 37° overnight.

Original rotation $[\alpha]_D^{15^\circ}$ -34°

After 24 hours treatment with yeast $[\alpha]_D^{15^\circ}$ -42.5°

Attempts were also made to prepare derivatives from the syrup. Various small test experiments were fitted up as indicated below:-

I	0.1 g. syrup	II	0.1 g. syrup
	1 ml. water		1 ml. water
	1 ml. phenylhydrazine		1 ml. alcohol
			1 ml. phenylhydrazine

III 0.1 g. syrup
1 ml. alcohol
1 ml. methylphenylhydrazine

The above test solutions were placed in a refrigerator for 48 hours. From I no derivatives were obtained even on adding excess alcohol. From II a very small precipitate was obtained on adding excess alcohol, while III gave a white crystalline methylphenylhydrazone m.p. 172°. Yield 0.098 g. This showed no depression of melting point on mixing with authentic fucose methylphenylhydrazone and was therefore considered to be fucose methylphenylhydrazone.

Elimination of fucose from the syrup using methylphenylhydrazine.

To the syrup (2.0818 g.) were added water (20 ml.), alcohol (20 ml.), methylphenylhydrazine (3 ml.) and glacial acetic acid (5 drops). The mixture was left in the refrigerator for several days and then filtered, and the precipitate washed and weighed. Yield 1.9147 g., corresponding to 62.9% fucose in the syrup. A test experiment with fucose (0.2038 g.) gave a yield of methylphenylhydrazone (0.2917 g.) corresponding to 95% of the theoretical amount.

After/

After extraction with chloroform to remove excess methylphenylhydrazine, the filtrate and washings from the fucose methylphenylhydrazone were evaporated to a volume of about 20 ml., and the remaining sugars in the syrup recovered by the method of Lüdtke (12). Ethanol (30 ml.), and freshly distilled benzaldehyde (4 ml.) were added and the solution refluxed for 4 hours. After keeping the solution at 0° overnight, the benzaldehyde methylphenylhydrazone was filtered off, washed thoroughly with water, and the alcohol removed from the filtrate and washings by evaporation. The aqueous solution was washed twice with ether, decolourised with charcoal, and evaporated in vacuo at 35° to give a pale yellow syrup. (Yield 0.714 g.). A fucose estimation on the syrup by the periodic acid oxidation method showed an apparent fucose content of 6.1%

Examination of "fucose-free" syrup.

1. Rotation: $[\alpha]_D^{15°}$ +8.4° in water, (c, 3.1).

2. The syrup was reducing to Fehling's and Barfoed's reagents.

3. The Seliwanoff and selenium dioxide tests were negative.

4. The Bredereck test gave a blue colour, though not/

not of the same intensity as that given by a sample of fructose.

5. The syrup gave no precipitate with alkaline hypoiodite, even on heating.

6. Uronic acids appeared to be present only as a trace, a pale violet colour being obtained with the naphthoresorcin test.

Test for mannose in "fucose-free" syrup (17).

To the syrup (0.5 g.) was added 3 ml. of water and 3 ml. of a mixture made up as follows:-

25 ml. phenylhydrazine
100 ml. absolute alcohol
3 ml. glacial acetic acid.

The solution was added and the syrup allowed to stand overnight, but no precipitate appeared.

Oxidation of "fucose-free" syrup with alkaline hypoiodite.

Into a colourless stoppered flask was weighed out 0.0618 g. of syrup and 30-40 ml. of 0.1 N sodium hydroxide was added. After diluting with water (200 ml.), an excess (20 ml.) of 0.1 N iodine was added and the solution allowed to stand for 60 minutes at room temperature. The solution was then neutralised with 2N sulphuric acid and the excess iodine/

iodine titrated with 0.1 N sodium thiosulphate using starch solution as an indicator. A blank experiment with the reagents was carried out at the same time.

Weight of syrup used 0.06180 g.

Weight of aldose present in syrup 0.03317 g.

(Volume of 0.1 N iodine required for oxidation 3.652 ml.)

% aldose in syrup 53.66 (as hexose)

But 6.1 % of the syrup is fucose which would react with the hypoiodite.

164 g. fucose ≡ 2 litres N iodine

0.00377 g. ≡ 0.463 ml. 0.1 N iodine

Titration due to other aldose is 3.189 ml. 0.1 N iodine

And weight of syrup oxidised is 0.05803 g. (after eliminating fucose)

% aldose in syrup (calculated as hexose) 49.5 %

ATTEMPTS TO DECOMPOSE FUCOSE METHYLPHENYLHYDRAZONE.

A. Using benzaldehyde.

In order to obtain some pure fucose, an attempt was made to decompose the methylphenylhydrazone. The usual method of Lüdtke (12) was adopted. The methylphenylhydrazone (1.8 g.), together with alcohol (150 ml.) and benzaldehyde (4.5 ml.) was refluxed for 5 hours on a boiling water-bath. After allowing to/

to stand at 0° overnight, the solution was filtered, but the precipitate was found on examination to be unchanged fucose methylphenylhydrazone. The experiment was repeated several times, but in all cases the fucose methylphenylhydrazone remained unchanged, probably due to its relative insolubility in alcohol.

B. Using p-nitrobenzaldehyde.

A series of preliminary experiments was first carried out, using various acids. Four trial experiments, each consisting of 50 mg. fucose methylphenylhydrazone, 150 mg. p-nitrobenzaldehyde and 5 ml. alcohol were set up and acids were added as follows:-

I 1 ml. dilute hydrochloric acid
II 1 ml. dilute sulphuric acid
III 50 mg. benzoic acid
IV 1 ml. concentrated hydrochloric acid.

Numbers I, II and III were refluxed on the water-bath for half an hour, while number IV was left standing for the same period of time. On examination, it was found that I and II were dark red in colour, III was light red and IV an intermediate shade of red.

Accordingly, a larger scale decomposition was attempted. To the fucose methylphenylhydrazone (1.12 g.) were/

were added alcohol (50 ml.), dilute sulphuric acid (10 ml.), and p-nitrobenzaldehyde (3 g.). The mixture was refluxed for two hours, after which water was added till the solution was just turbid. After neutralisation with barium carbonate, the solution was filtered, the residue extracted with alcohol, and filtrate and washings evaporated to a small volume. The resulting solution was extracted thoroughly three times with chloroform (50 ml.), and the aqueous fraction evaporated to dryness. A small quantity of a syrup was obtained, but it was found that this had no rotation. On adding alcohol a white solid, containing barium, was obtained.

C. Using p-nitrobenzaldehyde under pressure.

Fucose methylphenylhydrazone (1.5 g.), alcohol (50 ml.), benzoic acid (1.5 g.) and p-nitrobenzaldehyde (2.5 g.) were placed in a stout glass bottle with a screw stopper and heated in steam for 4-5 hours. The resulting red-coloured solution was diluted with a considerable excess of water and the solution evaporated *in vacuo* at 30° till all the alcohol had distilled off. The red precipitate which appeared was filtered, washed, and the filtrate and washings extracted four times with chloroform (250 ml.). On testing the solution it was found to be reducing.

From/

From the rotation of the solution it was estimated that about 15% of the fucose methylphenylhydrazone had been decomposed.

D. Using sulphuric acid.

To 1 g. of fucose methylphenylhydrazone was added 50 ml. of N sulphuric acid and the solution was heated on a boiling water-bath. After two hours, however, the solution was observed to have charred.

The experiment was repeated using 0.5 N sulphuric acid, and again darkening occurred, though to a slighter extent. The solution was neutralised with barium carbonate, filtered and extracted with chloroform, but, on evaporating the aqueous fraction, no fucose was obtained.

E. Using hydrochloric acid.

The method used was similar to that described by Gunther and Tollens (13), for the decomposition of fucose phenylhydrazone. To the methylphenylhydrazone (1.56 g.) was added 7.0 g. of hydrochloric acid (S.G. 1.19). To the solution, after standing overnight at room temperature, an equal volume of water was added, followed by 7.0 g. of lead carbonate. The filtrate from the lead chloride thus precipitated, was made alkaline with barium hydroxide solution, and freed from methylphenylhydrazine and other side products/

products by three extractions with ether. After treatment with charcoal, carbon dioxide was passed through the solution, which was then filtered and evaporated to half its volume. The solution was mixed with two volumes of alcohol, and one of ether, and silver sulphate was added. After shaking and filtering, the solution was evaporated but again no fucose was obtained.

Paper chromatogram examination of the hydrolysed polysaccharide.

A small quantity of the polysaccharide D was hydrolysed with 2% sulphuric acid for 8 hours, the solution neutralised, and a small spot of the solution placed on a paper chromatogram fitted up as described by Partridge (15), using as a solvent a mixture of n-butanol (40 parts), ethanol (10 parts) and water (50 parts), (the aqueous layer being placed in the bottom of the bell-jar and the upper layer in the trough). The results, after comparison with standards, indicated the presence of fucose in quantity, together with a small amount of what appeared to be a hexose.

DISCUSSION.

As a result of a series of sulphate estimations on the dried, ground specimens of various types of Scottish seaweeds, it was found that *Fucus vesiculosus* and *Ascophyllum nodosum* had the highest sulphate content of the samples examined. Since fucoidin is a carbohydrate sulphuric acid ester, it was considered that an estimation of the sulphate content of these seaweeds might give an idea of their relative suitabilities as sources of fucoidin. Owing to difficulty in obtaining quantities of *F. vesiculosus* and *A. nodosum*, however, many of the experiments were carried out on fucoidin extracted from *L. digitata* or *L. Cloustoni*, though the final large-scale extraction of the polysaccharide was carried out on dried samples of *F. vesiculosus* collected over a period of several months.

Lunde, Heen and Öy, while recognising that the purification of fucoidin was a matter of extreme difficulty, did not appear to encounter any trouble in the extraction of their samples, merely collecting the exudate from fresh fronds of *L. digitata* suspended in the atmosphere. It was found in the course of their experiments already described that, while fresh fronds were undoubtedly sticky, only

minute/

minute quantities of polysaccharide could be collected in this manner. Moreover, all cold water extractions carried out gave very low sulphate figures, suggesting the presence of another polysaccharide (probably soluble alginates) as well as fucoidin. Lunde's method could hardly avoid the contamination of his specimens with alginates though he did not appear to obtain any uronic acids in his product.

Kylin (4) claimed that fucoidin was precipitated by basic lead acetate, so, in an endeavour to precipitate the polysaccharide and avoid the lengthy process of concentrating <u>in vacuo</u> the large volumes of extract obtained, various experiments were carried out with this reagent. It was found, however, that the products obtained, though they had sulphate contents higher than those of previous precipitates obtained by simple extraction, were fairly impure. Moreover, the elimination of the excess lead acetate proved at first to be rather a difficult matter as precipitation of the lead as lead sulphide resulted in the formation of colloidal solutions. This was overcome, however, by precipitating the lead as lead sulphate with sulphuric acid.

Since a large proportion of the impurities present was probably composed of soluble alginates, methods/

methods of eliminating these prior to the precipitation of fucoidin were considered.

Eventually the most satisfactory method of obtaining the polysaccharide was found to be a hot water extraction which also removed the soluble alginates from the seaweed. These were satisfactorily eliminated by precipitation with lead acetate, the fucoidin remaining in solution. On making the solution alkaline with barium hydroxide, fucoidin was precipitated. Presumably a barium-lead hydroxide complex of some kind was formed, for, on adding dilute sulphuric acid, the barium and lead were precipitated as sulphates while the fucoidin, which went into solution, was still present as the calcium-magnesium salt.

Various methods of purification were tried, mostly with little success. Electrophoresis experiments, which, it was hoped, would show whether the polysaccharide obtained was a homogeneous substance, proved inconclusive, though migration did occur probably with some slight degradation of the polysaccharide. Fractional precipitation likewise proved of little use. By far the best results were obtained using Johns-Manville "Filter-Cel", the use of which raised the fucose content to 37.8% in the polysaccharide. This would appear to suggest that the/

the impurities encountered previously were partly due to colouring matter, and after such treatment, the polysaccharide was noticably lighter in colour, being a very pale buff, though the solutions of fucoidin obtained in this way had a brownish tinge.

The purified polysaccharide gave all the characteristic reactions of a carbohydrate ethereal sulphate already outlined on pages 3 - 5.

1. No precipitate of barium sulphate was obtained on adding barium chloride to an aqueous solution of the polysaccharide until after hydrolysis, showing that the sulphate group was combined and not ionised.

2. The metal in the polysaccharide, chiefly calcium, was ionised, as shown by the fact that an immediate precipitate of calcium oxalate was obtained on adding ammonium oxalate to an aqueous solution of fucoidin.

3. The ash, determined as sulphate, was a constant (24-24.2%), and analysis of the ash showed it to consist of calcium (24.5%), sodium (3.2%), potassium (0.8%), magnesium (1.0%) and sulphate (73.2%).

4. The total sulphate content of the polysaccharide (32.8%) was approximately double the sulphate content of the ash as sulphate (17.55%).

Comparing the analysis figures for the metals in the polysaccharide with those figures obtained by Lunde, it is observed that while the product he obtained/

obtained was chiefly a sodium and potassium salt, this is chiefly a calcium and sodium salt. Such differences are presumably due to the environment of the seaweeds. The fucose content of the final product obtained in this research (37.8%) is higher than that obtained by Lunde (33-37%) but the sulphate content (32.8%) never approached his figures (35.5-37.5%).

If the polysaccharide consisted entirely of fucose units, then, assuming one sulphate group were on each anhydrofucose unit, the sulphate content (the metal being $\frac{Ca}{2}$) would be 39.2%. Estimation of the fucose content in the polysaccharide gave a value of 37.8% but if each fucose residue was linked to two other residues, this figure would be reduced to 29.7%. This, together with 7.1% metals and 32.8% sulphate, means that the unidentified portion of the molecule comprises 30.4% of the whole. Accordingly, in such a molecule a sulphate group on each fucose unit would give a total sulphate content of 27.3%. It would thus appear from these calculations that either there is more than one sulphate group on some of the fucose residues or else some of the sulphate groups are attached to the unidentified portion.

The rate of removal of sulphate by aqueous sodium hydroxide (4%) was found to be very slow, indicating a straight hydrolysis without anhydro-ring formation.

Since the polyglucose, laminarin, is also present in the seaweeds L. digitata and L. Cloustoni, it was considered that fucoidin might contain a small percentage of laminarin and accordingly estimations of glucose according to the method of Sheehy, Brophy, Dillon and O'Muineachain (14) were carried out on the hydrolysed polysaccharide. The results of most of these experiments appeared to indicate that no glucose was present, but an examination of the "fucose-free" syrup did show indications of the presence of a little glucose by this method.

Contrary to reports of earlier investigators (4), the isolation of fucose phenylhydrazone from the polysaccharide after hydrolysis proved to be difficult, although the formation of the methylphenylhydrazone could be accomplished with ease. Accordingly, after hydrolysis of the polysaccharide with 2% sulphuric acid and neutralisation of the resulting solution with barium carbonate, a syrup was obtained containing 61.7% fucose which was eliminated by the formation of fucose methylphenylhydrazone. The

remaining/

remaining "fucose-free" syrup, which did however contain a small proportion (6.1%) of fucose, was recovered by the method of Lüdtke (12), the yield corresponding to 34.2% of the original hydrolysed syrup.

The "fucose-free" syrup had a small positive rotation, $[\alpha]_D^{15^\circ}$ +8.4°, and was reducing to Fehling's and Barfoed's reagents. Both the Seliwanoff and selenium dioxide tests for ketoses were negative, and, while the Bredereck test gave a blue colour, it did not appear to be the same as that given by a sample of fructose. With alkaline hypoiodite no iodoform was obtained, even on heating. The naphthoresorcin test for a uronic acid gave a faint violet colour, but only of such intensity as to suggest a trace of impurity (alginic acid) rather than a constituent. Oxidation with alkaline hypoiodite suggested the presence of an aldose. This reagent is used for the quantitative determination of aldehyde groups. With careful control of conditions, aldoses are converted practically quantitatively to aldonic acids, measurement of the amount of standard iodine consumed on titration giving the amount of aldose originally present.

$$R.CHO + I_2 + 3NaOH \longrightarrow R.COONa + 2NaI + 2H_2O$$

Oxidation/

Oxidation of the syrup in this manner gave results corresponding to 53.66% of an aldose (calculated as hexose), though this figure is lower (49.5%) if corrected for the fucose still present in the syrup.

An extremely useful method for estimating the purity of fucoidin was the modification by Cameron, Ross and Percival (7) of the method of Nicolet and Shinn (6), based on periodic acid oxidation of the fucose in the polysaccharide. When hydroxyl groups, or an amino and a hydroxyl group, are located on neighbouring carbon atoms, cleavage of the intermediate carbon-carbon bond occurs upon treatment with periodic acid:-

$$R'CHOH—CHOH—R + HIO_4 \longrightarrow R'CHO + R.CHO + HIO_3 + H_2O$$

$$R'CHNH_2—CHOH—R + HIO_4 \longrightarrow R'CHO + R.CHO + HIO_3 + NH_3$$

When more than two vicinal hydroxyl groups are available, the oxidation continues through this portion of the molecule with the formation of formic acid from secondary alcoholic groups, and formaldehyde from primary alcoholic groups.

$$CH_2OH—(CHOH)_n—CH_2OH \xrightarrow{HIO_4} n\,H.COOH + 2H.CHO$$

In the case of a methylpentose such as fucose, the terminal carbon atom will give acetaldehyde, which in the method already described is trapped as the bisulphite compound, decomposed with sodium bicarbonate, and titrated with standard iodine. Since one molecule of/

of acetaldehyde is obtained for each molecule of fucose, the percentage of fucose can thus be calculated.

In an endeavour to obtain specimens of pure L-fucose from the polysaccharide, attempts were made to decompose the methylphenylhydrazone. It was considered that the usual method of Lüdtke would be sufficient to decompose the compound, but although repeated attempts were made, this method proved to be completely unsuccessful. Further attempts were made using p-nitrobenzaldehyde in conjunction with various acids of various strengths, but only when heated with p-nitrobenzaldehyde and benzoic acid under pressure did the methylphenylhydrazone show any signs of decomposition, and then only to the extent of about 15%. Treatment of the compound with acids alone, although carried out with success on the phenylhydrazone by Gunther and Tollens (13), proved too drastic.

From the results of the above experiments, it is clear that the claim to have isolated a pure specimen of fucoidin can by no means be sustained. The portion of the polysaccharide not identified is considerable, and it cannot be stated whether several impurities are present or not. Nevertheless, it was hoped that by methylation a separation could be achieved/

achieved, or, failing that, a preliminary idea obtained of the mode of union of the L-fucose residues.

The possibility clearly existed that the polysaccharide was not a single substance, but a mixture of one containing fucose only (cf. Nelson and Cretcher (18)) and one or more other carbohydrate ethereal sulphates. Nevertheless, it seemed impossible to raise the fucose content above the figure already quoted.

SUMMARY.

1. Estimations on the dried, ground specimens of various types of Scottish seaweeds showed that, of the samples investigated, Fucus vesiculosus and Ascophyllum nodosum were the best sources of fucoidin, though there appeared to be no especially good source.

2. Various methods of extracting and purifying the polysaccharide have been tried, and a method which gives figures comparable to those given by Lunde (2) has been found. This involves a hot water extraction of the seaweed, precipitation of alginates with lead acetate and formation of a complex of fucoidin by adding barium hydroxide. The resulting product, after decomposition of the complex, dialysis and concentration, is purified by "Filter-Cel".

3. The polysaccharide $[\alpha]_D^{15^\circ}$ -117.8° obtained by hot-water extraction has been shown to be chiefly a calcium salt of a carbohydrate ethereal sulphate. Analysis of the ash as sulphate gives calcium 24.0-24.2% (5.8-5.9% of polysaccharide); sodium 3.2-3.6% (0.8-0.9%); magnesium 0.9-1.0% (0.2%); potassium 0.4-0.8% (0.10-0.2%); sulphate 72.0-73.2% (17.4-17.6%). Determination of total sulphate in the/

the polysaccharide gave 32.8%. Estimation of fucose gave 37.8% (29.7% as anhydrofucose), leaving 30.4% of an unidentified fraction.

4. The sulphate group of the polysaccharide strongly resisted hydrolysis by aqueous alkali, only 58.7% being hydrolysed in 72 hours.
5. The polysaccharide has been hydrolysed, and the fucose eliminated with methylphenylhydrazine, but no derivatives of the non-fucose portion have been obtained. This has not, however, been examined in detail, though hypoiodite oxidation suggests the presence of 49.5% aldose (calculated as hexose) in the "fucose-free" syrup.
6. Various attempts have been made to decompose fucose methylphenylhydrazone, none with much success.
7. A paper chromatogram investigation of the hydrolysed polysaccharide suggests the presence of a hexose, but this may be due to an impurity in the polysaccharide.

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P A R T II.

Investigation of Methylated Fucoidin.

EXPERIMENTAL.

Acetylation of the polysaccharide.

The polysaccharide D (2 g.) was dissolved in distilled water (30 ml.), pyridine (120 ml.) was added, and the pyridine-water azeotrope distilled off at 50°/15 mm. To ensure the complete elimination of water, the distillation was continued till the volume of the solution left in the flask was about 30 ml., when a brown gelatinous mass was obtained. Pyridine was added to bring the volume to 50 ml., the gelatinous solid adhering to the sides of the flask being scraped into the solution. Acetic anhydride (25 ml.) was then slowly added with shaking, the flask being cooled in water, and the mixture was left in the dark for two days. A flocculent white precipitate formed and this was centrifuged off, washed thoroughly with pyridine, alcohol and ether, and dried in a vacuum desiccator to give a white powder. Yield 2 g.

Determination of the acetyl value.

A weighed amount of the acetate (0.08159 g.) was placed in a flask containing 25 ml. of 0.1 N sodium hydroxide. The flask was stoppered, allowed to stand overnight, and the excess alkali titrated with/

with 0.1 N sulphuric acid, using phenolphthalein as indicator.

Found: $CH_3.CO$, 18.0%

Calc. for $C_6H_8O_7(COCH_3)S\frac{Ca}{2}$; $CH_3.CO$, 15.0%

Preliminary methylation experiments.

(a) Methylation of the acetate.

The acetate (1.5 g.) was dissolved in a little water (30 ml.) and to the solution were added, with mechanical stirring, dimethyl sulphate (1 ml.) and sodium hydroxide (2.5 ml.; 30%) every half hour at room temperature till a total of twelve additions had been made. The solution was then heated to 85° for half an hour, cooled, neutralised with glacial acetic acid, and dialysed against running water till free from sulphate and acid. The dialysed solution was evaporated at 45°/15 mm. to give a brown glass (OCH_3, 6.4%).

(b) Direct methylation of the polysaccharide D.

The polysaccharide D (1.0 g.) was dissolved in the minimum of water and the solution heated in a water-bath to 50° and kept at this temperature throughout the experiment. Additions of dimethyl sulphate (3 ml.) and sodium hydroxide (8.5 ml.; 30%) were made every two minutes with stirring, till eight additions had been made, after which a further eight additions/

additions of dimethyl sulphate (1.5 ml.) and sodium hydroxide (2.5 ml.; 30 %) were made at ten minute intervals. The solution was then heated to 85° for half an hour, cooled, neutralised with glacial acetic acid and dialysed till free from sulphate (and acid). On evaporation at 45°/15 mm. a brown glass was obtained (OCH_3, 7.5 %).

Direct methylation of a quantity of polysaccharide D.

To the polysaccharide D (10 g.) dissolved in the minimum of water, were made eight additions of dimethyl sulphate (30 ml.) and sodium hydroxide (84 ml.; 30 %) every two minutes, followed by eight additions of dimethyl sulphate (15 ml.) and sodium hydroxide (26 ml.) every ten minutes, the temperature being maintained at 50° during the course of the methylation. The experiment was then continued as in the previous methylation by the direct method. Further methylations were carried out till no increase in the methoxyl content of the polysaccharide was obtained.

	% OCH_3	
1st methylation	7.2	
2nd methylation	11.9	
3rd methylation	15.3	
4th methylation	15.5	Yield 6.8 g.

(Later/

(Later methylations gave approximately the same yield).

In an endeavour to ascertain whether or not the polysaccharide was fully methylated, an attempt was made to form an acetate. The product obtained gave $CH_3.CO$, 1.0% indicating within experimental error that the polysaccharide was fully methylated.

Inorganic analysis of the methylated polysaccharide.

The methylated polysaccharide (1.0043 g.; OCH_3, 15.5%) gave ash as sulphate (0.2126 g.; 21.2%). Analysis of the ash as sulphate, using the methods already indicated, gave the following:-

	Ash	Methylated Polysaccharide (calc. from ash)
Calcium (1)	24.13%	5.11%
Magnesium (2)	0.97%	0.21%
Potassium (3)	0.24%	0.05%
Sodium (4)	3.80%	0.80%
Sulphate	69.67%	14.75%
	98.81%	

The total sulphate was 25.93%.

$[\alpha]_D^{15°}$ -106.6° (c, 1.04 in water).

Hydrolysis of the methylated polysaccharide with 3% oxalic acid.

The methylated polysaccharide (5.01 g.) was hydrolysed at 100° with 3% oxalic acid (200 ml.) to constant/

constant rotation $[\alpha]_D^{15°}$ -29.1°. The solution was neutralised with barium carbonate, filtered, and the residue extracted several times with water. A syrup mixed with barium carbonate or sulphate was obtained on evaporation. This was extracted with alcohol and the filtered extracts evaporated at 40°/15 mm. to give a yellow syrup. Yield 3.3 g.; $[\alpha]_D^{13°}$ -54.0° (c, 1.11 in water); OCH_3, 20.4%.

Estimation of fucose by periodic acid oxidation (5) gave 30.4% as the apparent fucose content, or 33.0% calculated as monomethyl fucose. (See Discussion, page 137).

Treatment of syrup with 1% methyl alcoholic hydrogen chloride.

The sugar (0.082 g.) was dissolved in 1% methanolic hydrogen chloride (10 ml.), allowed to stand at room temperature (15° C) and the rotation was taken at intervals. $[\alpha]_D^{15°}$ -42.7° (initial value); -39.6° (1 hour); -36.2° (4 hours); -31.8° (7 hours); -29.3° (17 hours); -26.9° (22 hours); -24.4° (40 hours); -24.4° (52 hours, constant value).

The fall in the specific rotation was taken as a possible indication of furanoside formation (cf. the behaviour of D-galactose (6) and L-fucose (page 112)), and would appear to indicate the presence of free hydroxyl/

hydroxyl groups on position C_4 in at least some of the methylated sugars.

Formation and distillation of methylglycosides from the methylated sugars.

The syrup (2.5 g.) was refluxed with 2% methanolic hydrogen chloride (150 ml.) until no reducing action was obtained (5 hours). The resulting solution was neutralised with silver oxide and silver carbonate, filtered, and the residues extracted thoroughly with warm alcohol. The extracts were then evaporated to give a yellow syrup (2 g.).

A portion of this (0.5 g.) was transferred to a small distilling flask and distilled in a high vacuum. A clear syrup which appeared to distil as one fraction was obtained. Yield 0.38 g. Bath temp. 125-150°/0.1 mm.; $n_D^{11°}$ 1.4717; OCH_3, 29.8%.

Hydrolysis of methylglycosides with 0.5 N sulphuric acid and formation of osazone.

A portion of the distilled syrup (0.137 g.) was hydrolysed with 0.5 N sulphuric acid (10 ml.) till a constant rotation was obtained $[\alpha]_D^{15°}$ -57.7° (7 hours). After neutralisation with barium carbonate, the solution was worked up in the usual way, and evaporated to give a syrup.

In an endeavour to find out whether or not there was/

was a methoxyl group on C_2 of the methylated fucose present, an attempt was made to form an osazone from the syrup. The syrup was dissolved in water (2 ml.) and phenylhydrazine (0.5 ml.) and acetic acid (3 drops) were added. On placing the solution in a boiling water-bath for 3-4 hours a brown solid was obtained, which was filtered off, washed well with water and dried. m.p. 178-179° (decomp.); OCH_3, 9.2%

Calc. for a monomethyl osazone of fucose $C_{19}H_{24}O_3N_4$: OCH_3, 8.7%

Attempted preparation of acetone compounds of the methylglycosides.(7).

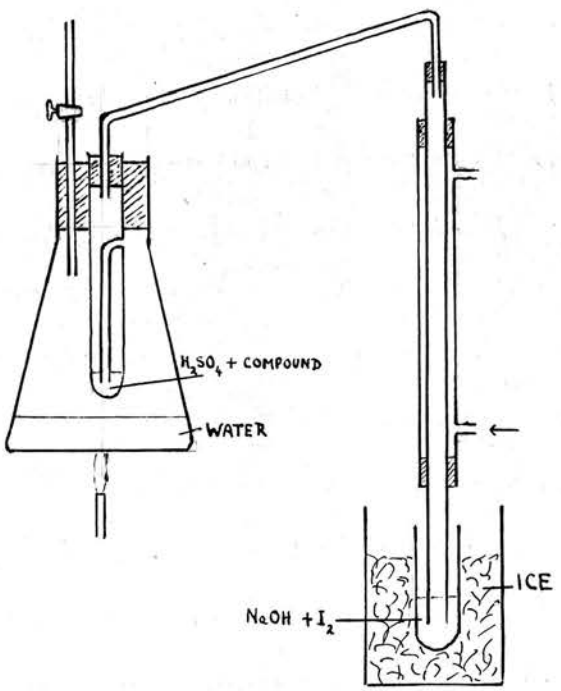
The mixture of methylglycosides (1.7 g.) was dissolved in dry acetone (300 ml.) and to this was added anhydrous copper sulphate (40 g.), and five drops of acetaldehyde. After shaking for three days, the solution was filtered, the precipitate extracted with acetone (1 litre) and the filtrate and extracts evaporated in the presence of a little barium carbonate. To the residue was added chloroform (250 ml.) and after standing overnight, the solution was heated on the water-bath, filtered, evaporated, transferred to a small distilling flask, and distilled in a high vacuum.

Fraction	Bath Temp.	$n_D^{13°}$	Yield	
X	90-100°	1.4567	0.31 g.	
Y	110-120°	1.4678	0.72 g.	(partially crystalline)
Z	120-135°	1.4694	0.55 g.	

Micro-determination of acetone. (8)

(modified from Bell and Harrison (9).)

The apparatus consists of a 500 ml. wide-mouthed conical flask with a bubbler tube and an escape stop-cock fitted in the bung. The bubbler is connected to a condenser leading to a 100 ml. test-tube.



N sodium hydroxide (10 ml.) and 0.03 N standard iodine (25 ml.) are mixed in the receiver cooled in ice.

N sulphuric acid (3.0 ml.) is placed in the bubbler and the glass capsule containing 5-6 mg. of acetone compound is dropped into it. The boiler is heated with a small flame so that an initial slow current of steam passes through the bubbler. After five minutes, the rate of heating is increased to drive out the last traces of acetone. This is continued for 30 minutes, and for a further 5 minutes on removing the condenser from the liquid. After rinsing the walls of the receiver and foot of the condenser, the sodium hydroxide is neutralised with N sulphuric acid (15 ml.), and the iodine titrated with 0.03 N sodium thiosulphate, using starch solution as an indicator. A blank experiment with the reagents is treated similarly.

$$\% \text{ Acetone} = \frac{(\text{Blank-titration})(\text{normality of } Na_2S_2O_3)(0.9666)}{\text{Weight of material}}$$

Fraction	% Acetone	$\% OCH_3$
X	7.2	43.6
Y	3.5	30.4
Z	2.5	27.2

ATTEMPTED SEPARATION OF THE METHYLATED METHYLGLYCOSIDES BY CHROMATOGRAPHIC ADSORPTION (10).

The methylated polysaccharide (8.9 g.) was heated at 100° with 3% oxalic acid (400 ml.) for 17 hours, the air in the flask being displaced by a slow stream of nitrogen. The resulting solution was neutralised and worked up in the usual way to give a syrup which was converted into the corresponding methylglycosides by treatment with 2% methanolic hydrogen chloride as before. Yield 5.8 g.

The syrup was dissolved in a 1:1 chloroform: petrol-ether (40-60°) mixture and introduced into a column of activated alumina (Peter Spence and Sons; Grade H) 30 cm. x 2 cm. suspended in the same solvent. This mixture was used to develop the column and portions were collected and evaporated under diminished pressure. When little or no syrup was obtained in the fractions, the proportion of chloroform to petrol-ether was gradually increased, after which mixtures of chloroform and methanol and finally pure methanol were used. When no more syrup could be recovered in this way, the alumina was placed in a Soxhlet extractor and extracted with methanol for several hours. Finally the alumina was extracted with water.

Fraction	Vol. of Soln.	Yield	Temp.	n_D	% OCH_3
	Chloroform:petroleum ether 1:1				
1	30 ml.	0.003 g.	13°C		
2	30	0.018	13°	1.4578	
3	60	0.140	13°	1.4602	45.8
4	60	0.301	13°	1.4604	
5	30	0.324	13°	1.4600	45.4
6	30	0.216	13°	1.4590	
7	30	0.034	13°	1.4583	
		1.036			
	Chloroform:petroleum ether 2:1				
8	30	0.015	13°	1.4582	
9	60	0.017	11°	1.4584	
10	60	0.014	11°	1.4580	
11	100	0.016	11°	1.4578	
12	100	0.012	12°	1.4580	
		0.074			
	Chloroform:petroleum ether 3:1				
13	30	0.012	10°	1.4580	
14	30	0.006			
15	100	0.010			
		0.028			
	Chloroform				
16	30	0.004			
17	60	0.008			
18	100	0.010			
		0.022			

Fraction	Vol. of Soln.	Yield	Temp.	n_D	% OCH_3
		Chloroform:methanol 3:1			
19	30 ml.	0.040 g.	13°C	1.4649	
20	60	0.061	12°	1.4652	
21	30	0.104	12°	1.4669	31.9
22	30	0.238	12°	1.4668	31.7
23	30	0.479	12°	1.4670	30.9
24	30	0.482	12°	1.4670	32.3
25	30	0.084	12°	1.4672	
26	60	0.078	13°	1.4670	
27	60	0.072	13°	1.4670	
		1.638			
		Chloroform:methanol 2:1			
28	30	0.042	13°	1.4670	
29	60	0.040	13°	1.4678	
30	60	0.041	13°	1.4681	
31	60	0.048	13°	1.4680	
		0.171			
		Chloroform:methanol 1:2			
32	30	0.050			
33	30	0.054	11°	1.4672	
34	30	0.058	11°	1.4675	
		0.162			

Fraction	Vol. of Soln.	Yield	Temp.	n_D	$\%OCH_3$
		Methanol			
35	30 ml.	0.045 g.			
36	30	0.045			
37	30	0.078			
38	30	0.084	13°C	1.4691	
39	30	0.094			
40	60	0.098			
41	60	0.142	12°	1.4688	33.1
42	60	0.106			
43	60	0.082			
44	60	0.045	11°	1.4684	
45	60	0.040			
46	100	0.024			
47	100	0.018			
		0.901			

Amount recovered from Soxhlet extraction of alumina with methanol 0.878 g.; OCH_3, 17.4%

Amount recovered from extraction of alumina with water 0.182 g.; OCH_3, 16.8%

Total amount recovered 5.094 g.

% recovery : 88%

Fractions 1 - 7 were combined to give a syrup which will be called Fraction I. Since crystals showed signs of appearing in Fractions 8 and 10, Fractions/

Fractions 8 - 18 were combined and evaporated , when part of the syrup obtained crystallised giving Fraction II. Fraction 22 also showed signs of crystallising, so it was decided to combine Fractions 19 - 28, giving Fraction III. The remainder of the fractions were also combined to give Fraction IV.

The fractions may thus be summarised:-

Fraction I	1.036 g.	
II	0.124 g.	(partially crystalline)
III	1.680 g.	(partially crystalline)
IV	1.192 g.	
Methanol extract	0.878 g.	
Water extract	0.182 g.	
	5.092 g.	

The various fractions were then examined with a view to identifying the sugars and determining the position of the methoxyl groups in them. Experiments with paper chromatograms, though reported here for completeness, were actually done at a later stage of the research.

Examination of Fraction I.

The fraction (0.97 g.) was transferred to a small distilling flask and distilled in a high vacuum. Two fractions were collected:-

Fraction	Bath temp.	Yield	$n_D^{13°}$	% OCH_3
Ia	75-80°/0.03 mm.	0.478 g.	1.4549	46.6
Ib	90-110°/0.03 mm.	0.385 g.	1.4601	47.7

Calc. for a dimethyl methylfucoside $C_6H_9O_2(OCH_3)_3$: OCH_3, 45.1 %

Later experiments using the paper chromatogram method of identification indicated that the two fractions were identical.

Formation of 2:3:4-trimethyl α-methyl-L-fucoside from Fraction Ia.

Part of Fraction Ia (0.2 g.) was dissolved in methyl iodide (25 ml.) and silver oxide (3 g.) added in one-sixth portions every half-hour with occasional shaking, the solution being kept at 40°. The methylation was allowed to proceed overnight, and the solution was then filtered, the residues exhaustively extracted with hot chloroform, and the filtrates and extracts evaporated to a syrup, which was methylated once more as above. On evaporation after the second methylation a white crystalline compound which sublimed *in vacuo* was obtained. The crystals (0.18 g.) were long and needle-shaped. m.p. 84-89°. A comparison with authentic 2:3:4-trimethyl α-methyl-L-fucoside was carried out.

m.p. of authentic specimen 86-92°

Mixed m.p. 84-88°

$[\alpha]_D^{14°}$ -191.8° (c, 1.08 in water)

Found: OCH_3, 56.1%

Calc. for $C_{10}H_{20}O_5$: OCH_3, 56.3%

It thus appeared almost certain that the derivative obtained was 2:3:4-trimethyl α-methyl-L-fucoside.

Periodic acid oxidation of Fraction Ib and isolation of D(-)-dimethoxysuccinamide.

The syrup (0.20g.) was hydrolysed for 8 hours at 100° with 0.1 N sulphuric acid (50 ml.) $[\alpha]_D^{15°}$ -61° ⟶ -1°, and the solution was worked up in the usual manner. To the syrup obtained was added 0.5 M periodic acid (20 ml.) and the solution was allowed to stand for 20 hours at room temperature. After thorough aeration to remove any acetaldehyde formed, barium chloride (1.22 g.) was added to the solution, followed by excess barium carbonate. The residue, after filtering, was extracted with a small quantity (20 ml.) of water. Bromine (3 ml.) was added, and the solution allowed to stand for three days, after which the bromine was removed by aeration, the solution neutralised with silver carbonate, and hydrogen sulphide passed through the filtrate to remove the silver. On evaporation under reduced pressure, a small quantity of syrup, together with some inorganic salt, was obtained.

The residue was refluxed with 2½% methanolic hydrogen chloride (50 ml.) for five hours, the solution neutralised with silver oxide and silver carbonate, and after several extractions of the residue with alcohol, the filtrate was evaporated to dryness. A syrup mixed with some white inorganic salt was obtained, which was extracted with ether, the extract evaporated, and methanolic ammonia added to the syrup. After standing three days at 0°, the solution was evaporated, when a crystalline solid mixed with syrup was obtained. The crystals (12 mg.) were washed carefully with alcohol and dried.

Examination of the crystals showed them to be long and needle-shaped. m.p. 262-263°.

Various melting-points and mixed melting-points were carried out.

m.p. of D(-)-dimethoxysuccinamide	265-268°
m.p. of i-dimethoxysuccinamide	269-271°
Mixed m.p. of amide with:- D(-)-dimethoxysuccinamide	265-268°
i-dimethoxysuccinamide	235-240°

Rotation of amide $[\alpha]_D^{15}$ -92° (c, 2.01 in water).
(cf. D(-)-dimethoxysuccinamide $[\alpha]_D$ -94°).

Found: OCH_3, 34.8%

Calc. for $C_6H_{12}O_4N_2$: OCH_3, 35.3%

The derivative thus appeared to be D(-)-dimethoxy-succinamide.

Examination of Fraction II.

After drying the crystals on a tile and recrystallising them from petrol-ether (40-60°), only a very small quantity (12 mg.) was obtained, so examination had to be restricted to a melting-point, analysis and the rotation.

m.p. 49-51°. $[\alpha]_D^{13°}$ -190° (c, 1.43 in water).

Found: C, 52.3; H, 8.6; OCH_3, 44.3%

Calc. for a dimethyl methylfucoside $C_9H_{18}O_5$:

C, 52.4; H, 8.7; OCH_3, 45.1%

The compound thus appeared to be probably a dimethyl α-methyl-L-fucoside.

Examination of Fraction III.

Attempted purification by chromatographic adsorption.

Fraction III was partially crystalline, and it was considered that if passed through a chromatographic column some impurities might be removed and a greater proportion of crystals obtained. Accordingly, the fraction (1.62 g.) was dissolved in a mixture of 3:1 chloroform:methanol and the solution passed through a small column of alumina (20 cm. x 1 cm.). The same solvent was used to develop the column.

Fraction	Vol. of soln.	Yield	Temp.	n_D
1	30 ml.	0.020 g.	13°C	1.4650
2	30	0.028	13°	1.4650
3	60	0.046	13°	1.4652
4	60	0.081	13°	1.4668
5	30	0.106	11°	1.4670
6	30	0.138	10°	1.4672
7	30	0.207	10°	1.4678
8	30	0.277	11°	1.4672
9	30	0.166	11°	1.4674
10	30	0.132	11°	1.4674
11	30	0.097	12°	1.4674
12	60	0.098	12°	1.4682
13	60	0.044	12°	1.4682
14	60	0.046	12°	1.4688
15	60	0.010		
		1.496		

Fraction 6 crystallised and the crystals, hereafter called Fraction V, were placed on a tile to remove traces of syrup. The remainder of the fractions, 1-5 and 7-15 appeared to be identical, and were accordingly combined to give Fraction VI.

Examination of Fraction IV.

The methoxyl content of this fraction (33.1%) indicated that it was probably composed of a mixture of/

of methylated sugars, so it was decided to extract the mixture with chloroform. The fraction (0.619 g.) was dissolved in water (25 ml.) and after the rotation had been taken $[\alpha]_D^{15°}$ -59.8°, the solution was shaken several times with chloroform (50 ml.). The chloroform extracts IVa, and the aqueous layer IVb, were then both evaporated under diminished pressure.

Fraction IVa	0.18 g.	OCH_3, 36.5 %
IVb	0.43 g.	OCH_3, 29.8 %

Formation of 2:3:4-trimethyl α-methyl-L-fucoside from both fractions.

The syrup IVa was dissolved in methyl iodide and methylated three times by the Purdie method as already described. As before, long needle-shaped crystals were obtained (0.12 g.; m.p. 84-90°), showing no depression of melting-point on mixing with authentic 2:3:4-trimethyl α-methyl-L-fucoside.

Complete methylation of part of Fraction IVb also gave the same derivative, m.p. 82-87°, OCH_3, 55.8 % $[\alpha]_D^{14°}$ -190° (c, 1.5 in water).

Hydrolysis and examination of Fraction IVb.

The remainder of Fraction IVb (0.22 g.) was hydrolysed with 0.1 N sulphuric acid at 100° for 16 hours, after which the product was neutralised with barium/

barium carbonate and worked up in the usual way to give a syrup.(0.18 g.). OCH_3, 16.9%.

$[\alpha]_D^{15°}$ -88° (c, 0.55 in water).

A drop of a solution of the syrup was placed on a paper chromatogram, fitted up as described by Partridge (11), using as solvent a butanol (40 parts), water (50 parts), alcohol (10 parts) mixture. Comparison with suitable standards - fucose, 2-methyl fucose (see page 125) and digitalose (see page 128) - indicated that the syrup was composed of 3-methyl fucose together with a little fucose.

Treatment of hydrolysed IVb with 1% methanolic hydrogen chloride.

The fraction (0.1020 g.) was dissolved in 1% methanolic hydrogen chloride (10 ml.) and the rotation of the solution taken at intervals.

$[\alpha]_D^{15°}$ -90.0° (0 hours); -84.7° (2 hours); -80.1° (4 hours); -74.6° (6 hours); -57.2° (10 hours); -28.0° (20 hours); -24.7° (22 hours); -24.7° (24 hours); -18.5° (27 hours); -16.7° (43 hours); -12.0° (60 hours); -10.8° (71 hours); -8.2° (89 hours); -4.1° (97 hours, constant).

By way of comparison, L-fucose was treated in the same way with 1% methanolic hydrogen chloride.

$[\alpha]_D^{15°}$ -86.1° (0 hours, c, 0.8); -62.0° (2 hours);

-55.6° (4 hours); -50.2° (6 hours); -27.0° (10 hours); -2.0° (20 hours); +2.1° (22 hours); +3.8° (24 hours); +4.2° (27 hours, constant).

The results of this experiment afforded further confirmation that position C_4 was occupied by a free hydroxyl group.

Attempted preparation of an osazone from the methanolic-hydrogen chloride solution of hydrolysed IV b.

The solution was evaporated to dryness, dissolved in a little water, and two drops of phenylhydrazine and a little sodium acetate added. On heating on the water-bath for several hours, a dark brown tar was obtained. The product was filtered off and washed well with water to give a dark brown solid, m.p. 172-176° (decomp.). A qualitative examination showed that methoxy was present which was taken as an indication of the presence of a free hydroxyl group on C_2.

Examination of Fraction V.

The fraction, after drying on a porous tile, was recrystallised from ethyl acetate and analysed, m.p. 130-132°; $[\alpha]_D^{14°}$ -173.1° (c, 0.4 in water).

Found: C, 50.3; H, 8.4; OCH_3, 32.1%

Calc. for $C_8H_{16}O_5$: C, 50.0; H, 8.3; OCH_3, 32.3%
(a monomethyl methylfucoside)

The remainder of the fraction was hydrolysed with 0.1 N sulphuric acid for 8 hours. After treatment in the usual way, a small quantity of syrup was obtained.

$[\alpha]_D^{15°}$ -94° (c,0.51 in water). OCH_3, 17.8%

On placing a spot of this syrup on a paper chromatogram with suitable standards for comparison - fucose, 2-methyl fucose and digitalose (3-methyl D-fucose) - it appeared to be entirely 3-methyl fucose. The crystalline fraction was therefore identified as 3-methyl α-methyl-L-fucoside.

Examination of Fraction VI.

Fraction VI was also hydrolysed with 0.1 N sulphuric acid and after working up, was examined by the paper chromatogram method. It appeared to be identical with Fraction V.

Found: OCH_3, 17.0%

Calc. $C_7H_{14}O_5$: OCH_3, 17.1%

Examination of fractions extracted by alcohol and by water.

Both fractions on keeping showed signs of crystallising, and eventually from the methanol-soluble fraction a quantity (0.2 g.) of crystals was obtained. These were recrystallised from ethyl acetate.

m.p. 154-156°. $[\alpha]_D^{15°}$ -197° (c, 1.1 in water).

Found: OCH_3, 17.2%

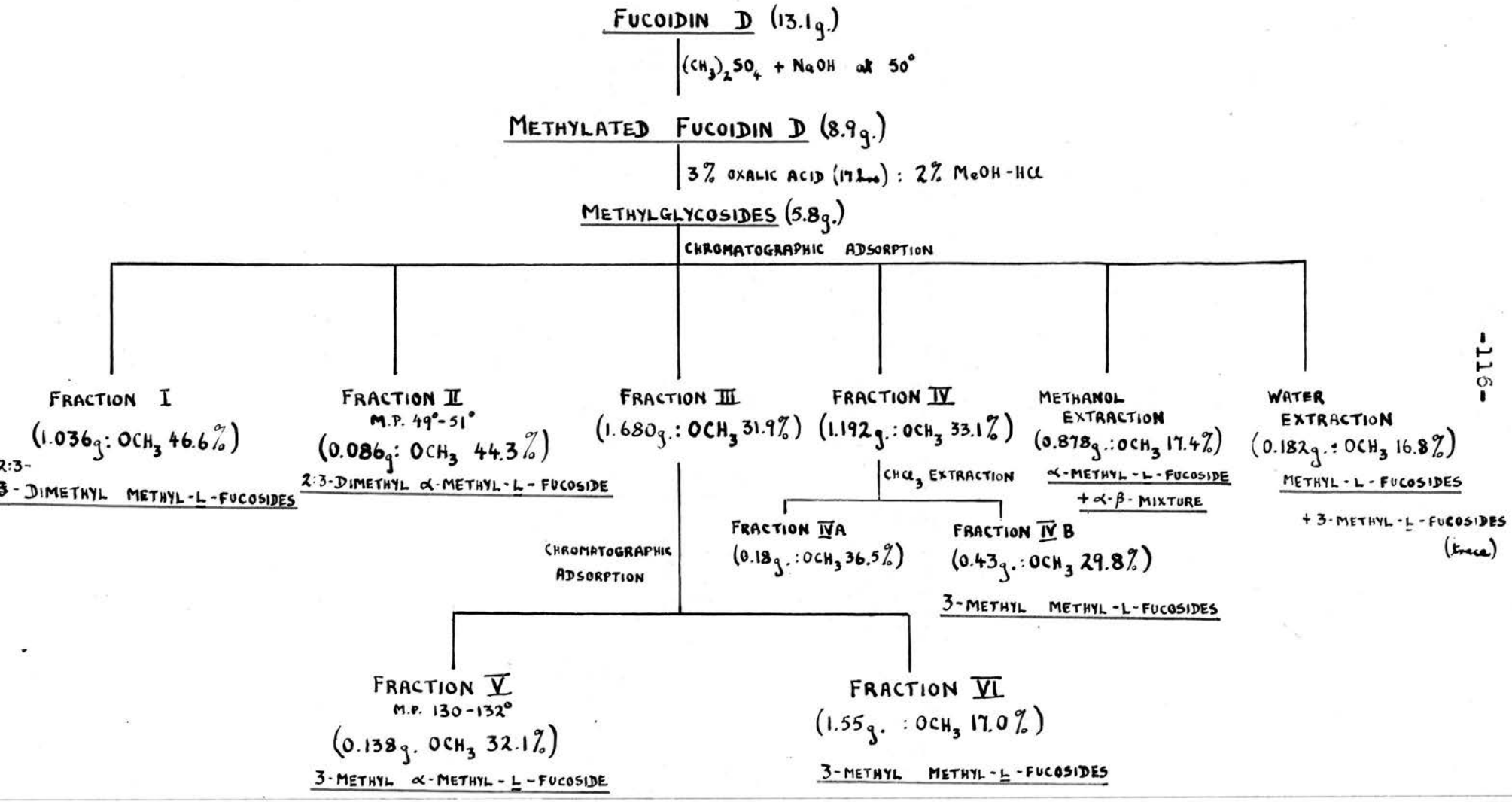
From these constants it appeared probable that the crystals were α-methyl-L-fucoside and on hydrolysis and examination by paper chromatography only fucose was obtained.

Both fractions were hydrolysed with 0.1 N sulphuric acid and examined by paper chromatography, when it was observed that they both appeared to be composed of fucose with a trace of 3-methyl fucose, and possibly a trace of a uronic acid.

After hydrolysis:-

	$[\alpha]_D^{13°}$	% Fucose
Alcohol fraction	-72.1° (c,0.91 in water)	93.1
Water fraction	-69.8° (c,0.84 in water)	87.8

A summary of the various fractions obtained in this separation is given overleaf.


FUCOIDIN D (13.1g.)
$(CH_3)_2SO_4$ + NaOH at 50°
METHYLATED FUCOIDIN D (8.9g.)
3% OXALIC ACID (171 ml) : 2% MeOH-HCl
METHYLGLYCOSIDES (5.8g.)
CHROMATOGRAPHIC ADSORPTION
FRACTION I
(1.036g: OCH_3 46.6%)
2:3-
3-DIMETHYL METHYL-L-FUCOSIDES
FRACTION II
M.P. 49°-51°
(0.086g: OCH_3 44.3%)
2:3-DIMETHYL α-METHYL-L-FUCOSIDE
FRACTION III
(1.680g.: OCH_3 31.9%)
FRACTION IV
(1.192g.: OCH_3 33.1%)
METHANOL EXTRACTION
(0.878g.: OCH_3 17.4%)
α-METHYL-L-FUCOSIDE
+ α-β-MIXTURE
WATER EXTRACTION
(0.182g.: OCH_3 16.8%)
METHYL-L-FUCOSIDES
+ 3-METHYL-L-FUCOSIDES
(trace)
$CHCl_3$ EXTRACTION
FRACTION IVA
(0.18g.: OCH_3 36.5%)
FRACTION IV B
(0.43g.: OCH_3 29.8%)
3-METHYL METHYL-L-FUCOSIDES
CHROMATOGRAPHIC ADSORPTION
FRACTION V
M.P. 130-132°
(0.138g. OCH_3 32.1%)
3-METHYL α-METHYL-L-FUCOSIDE
FRACTION VI
(1.55g. : OCH_3 17.0%)
3-METHYL METHYL-L-FUCOSIDES

ATTEMPTED SEPARATION OF THE METHYLATED GLYCOSIDES BY THE SOLVENT EXTRACTION METHOD (12).

A fresh quantity of fucoidin D was methylated four times by the Haworth method, (OCH_3, 15.8%). The methylated polysaccharide (8.68 g.), was hydrolysed at 100° for 8 hours with 0.5 N oxalic acid and the solution neutralised and extracted as before. Yield 6.0 g.

The syrup (5 g.) was refluxed with 2% methyl alcoholic hydrogen chloride (200 ml.) for 5 hours, neutralised with silver oxide and silver carbonate, and the extracts and filtrate evaporated. This syrup containing the mixture of glycosides was dissolved in water (20-30 ml.) and extracted in a solvent extraction apparatus with 40-60° petroleum-ether (200 ml.). When no more syrup could be obtained in this way, a chloroform extraction apparatus was used, and finally the residual aqueous solution was evaporated.

Fraction	Duration of extraction	Yield	Temp.	n_D
		Petroleum-ether 40-60°		
A	4½ hrs.	0.050 g.	13°C	1.4501
B	10½	0.270	13°	1.4498
C	15½	0.060	13°	1.4499
		0.380		

Fraction	Duration of extraction	Yield	Temp.	n_D
		Chloroform.		
D	4½ hrs.	1.80 g.	15°C	1.4610
E	23½	1.660	15°	1.4735
F	34	0.240	(partially crystalline).	

The aqueous solution was then evaporated, giving Fraction G.

Since Fractions A, B and C seemed identical, they were combined and distilled in a high vacuum, giving a clear syrup, Fraction A1 (0.28 g.; $n_D^{15°}$ 1.4442). Bath temp. 83-87°C/0.03-0.04 mm.

Thus, summarising the fractions obtained:-

Fraction	Yeild	$\%OCH_3$
A1	0.28 g.	49.8
D	1.80	40.0
E	1.66	28.6
F	0.24	30.9
G	0.97	16.4
	4.95	

Separation of Fraction D by chromatographic adsorption.

Since the methoxyl content of Fraction D (40.0%) appeared to indicate that it was composed of a mixture of mono- and dimethyl methylglycosides, the syrup (1.8 g.) was dissolved in chloroform and passed/

passed through a 20 cm. x 2 cm. column of activated alumina. After the column had been developed with chloroform, the solvent was changed to methanol.

Fraction	Vol. of soln.	Yield
	Chloroform	
1	30 ml.	0.040 g.
2	60 ml.	0.060 g.
3	60 ml.	0.127 g.
4	30 ml.	0.293 g.
5.	30 ml.	0.186 g.
6	30 ml.	0.090 g.
7	100 ml.	0.080 g.
8	100 ml.	0.014 g.
		0.890 g.
	Methanol	
9	60 ml.	0.023 g.
10	100 ml.	0.089 g.
11	100 ml.	0.173 g.
12	60 ml.	0.246 g.
13	60 ml.	0.255 g.
14	60 ml.	0.058 g.
15	100 ml.	0.027 g.
		0.871 g.

The chloroform fractions were combined and, since they appeared to contain a little wax, were distilled/

distilled (100-110°/0.04 mm.). On distillation a colourless syrup was obtained which, on standing in the refrigerator, crystallised, (Fraction D1). The methanol extracts were combined to give Fraction D2.

Fraction	Yield	$n_D^{16°}$	% OCH_3	Apparent % of fucose (page 137)
D1	0.79 g.	1.4590	46.33	32.4
D2	0.871	1.4669	31.14	34.2

Examination of Fraction D1. (OCH_3, 46.3%).

The syrup (0.3 g.) was hydrolysed at 100° for 9 hours with 0.1 N sulphuric acid (100 ml.) to give after the usual treatment a syrup (0.28 g.), $[\alpha]_D^{15°}$ +4.6° (c, 3.0 in water).

Found: OCH_3, 32.1%.

An attempt was made to prepare the anilide of the sugar. 19.68 mg. of aniline were added to 41 mg. of the sugar and the solution refluxed at 90° for 2 hours. After leaving in the refrigerator overnight the solution was evaporated at 15°/15 mm., when a syrup was obtained. This did not crystallise even when kept for several months.

Since the anilide showed no sign of crystallising, it was decomposed by heating with 1% sulphuric acid (10 ml.) for 1½ hours. The solution was cooled, neutralised with barium carbonate, filtered and the barium/

barium sulphate extracted with water. The filtrate and washings were then shaken several times with ether (50 ml.) to remove the aniline and the aqueous solution was evaporated at 50°/15 mm. The resulting syrup was extracted with alcohol and the solution again evaporated; a crystalline dimethyl fucose could not, however, be isolated.

Formation of the lactone of hydrolysed Fraction D1.

The syrup (0.18 g.) was dissolved in water (5 ml.), bromine (1.5 ml.) was added and the solution allowed to stand at room temperature for 30 hours (till a portion of the solution after aeration was found to be non-reducing). The bromine was removed by aeration and the solution neutralised with silver carbonate. After filtering, and extracting the silver residues, hydrogen sulphide was passed through till all the silver was precipitated. The silver sulphide precipitate was filtered off, extracted with hot water and filtrate and washings evaporated to dryness. Since there were still indications of the presence of silver salts, the residue was extracted with chloroform and after evaporation to a small volume, transferred to a small flask and distilled (130°/0.04 mm.). A clear syrup was obtained (0.1 g.)

Found: OCH_3, 34.8%

Calc. $C_8H_{14}O_5$: OCH_3, 32.6%

Rotation of the lactone.

The syrup (0.088 g.) was dissolved in water (5 ml.) and the rotation observed at intervals.

$[\alpha]_D^{12°}$ +9.2° (0 hours); +9.8° (½ hour); +10.7° (1 hour); +23.0° (3 hours); +27.5° (4 hours); +34.4° (8 hours); +36.8° (12 hours); +40.2° (15 hours); +46.9° (22 hours, constant).

The positive rotation indicates the presence of a dimethyl γ-L-fuconolactone. (cf. 2:3:4-trimethyl L-fuconolactone $[\alpha]_D$ -138° ⟶ -36° (13).

1 ml. of the rotation solution was titrated with 0.025 N sodium hydroxide, using phenolphthalein as indicator.

Vol. of 0.02713 N NaOH required 1.67 ml.

Theory for a dimethyl fuconolactone 1.79 ml.

Amide formation.

To the lactone (40.4 mg.) was added methanolic ammonia (2 ml.) and the solution was left in the refrigerator for 48 hours. On evaporation at 14°/15 mm. the entire solution crystallised. The white solid formed was recrystallised from petrol-ether (60-80°) m.p. 78-79°; $[\alpha]_D^{15°}$ +30.2° (c, 0.86 in water).

Found: OCH_3, 30.0 %

Calc. for amide of a dimethyl fuconic acid $C_8H_{17}O_5N$: OCH_3, 30.0 %

Weerman test on amide (14).

To the amide (30 mg.) was added sodium hypochlorite solution (2 ml.) and the mixture was left for 3 hours at 0°C. The solution was then saturated with sodium acetate, and a few drops of concentrated sodium thiosulphate added (till no hypochlorite was present on testing with starch-iodide paper). After filtering, 2 ml. of saturated semicarbazide hydrochloride were added, and the solution was left in the refrigerator overnight. No precipitate was obtained.

A similar experiment with gluconamide (48.21 mg.) gave a bulky white precipitate (16.92 mg.) of hydrazodicarbonamide, m.p. 255° (decomp.).

From this result it is clear that the hydroxyl group on C_2 is substituted by methoxyl.

Examination of Fraction D2. (OCH_3, 31.1 %)

The fraction (0.258 g.) was hydrolysed with 0.1 N sulphuric acid at 100° till the rotation was constant (8 hrs.: $[\alpha]_D^{15°}$ -29° ⟶ -19.4°). A portion (0.107 g.) of the syrup obtained after the usual treatment was dissolved in 10 ml. of 1% methanolic hydrogen chloride and the rotation taken at intervals. $[\alpha]_D^{14°}$ -18.2° (0 hours); -16.9° (1 hour); -13.9° (3 hours); -13.9° (5 hours); -12.7° (8 hours);

-10.2° (12 hours); -9.8° (18 hours); -8.6° (22 hours); -8.6° (26 hours); -8.6° (29 hours); -6.6° (46 hours); -2.2° (90 hours, constant).

This appears to indicate that the fraction has position C_4 occupied by a hydroxyl group (cf. pages 112-113).

Formation of the lactone of D2.

The remainder of the above hydrolysed fraction (0.091 g.) was dissolved in water (5 ml.) and oxidised with bromine in the manner already described. On finally evaporating to obtain the lactone a crystalline product, m.p. 132-136°, was obtained. Yield 47.7 mg.

Rotation of lactone of hydrolysed D2.

The lactone (47.7 mg.) was dissolved in water (5 ml.) and the rotation followed.

$[\alpha]_D^{14°}$ +25.1° (10 mins.); +36.8° (½ hr.); +46.0° (1 hour); +46.0° (2 hours); +47.4° (3 hours); + 50.3° (7 hours); +53.2° (12 hours); +60.8° (19 hours); +74.1° (32 hours); +74.8° (48 hours); +751° (62 hours, constant).

This appears to be a γ-lactone indicating that C_4 was occupied by a hydroxyl group in the methylated sugar.

Preparation of amide.

To the lactone (47 mg.) was added methanolic ammonia (2 ml.), and the solution was allowed to stand at 0° for 48 hours. The solution was then evaporated at room temperature to give a syrup, $[\alpha]_D^{15°}$ +17.2° (c, 0.61 in water).

Found: OCH_3, 16.7%

Calc. for $C_7H_{15}O_5N$: OCH_3, 16.1%

Weerman test on the amide.

A Weerman test was carried out on the syrup (40 mg.) as already described for the amide from the lactone of D1.

Weight of precipitate obtained 14.98 mg. (m.p. 253-255°).

This confirms that in the amide, position C_2 is occupied by a hydroxyl group.

PREPARATION OF DERIVATIVES FOR COMPARISON.

A. Synthesis of 2-methyl L-fucose (7).

L-fucose (0.1 g.) was dissolved in 2% methanolic hydrogen chloride and refluxed for 5 hours (till no reducing action was obtained). The solution was neutralised with silver oxide and silver carbonate, filtered, and the residues extracted with warm alcohol. The resulting solution was evaporated to give/

give a syrup.

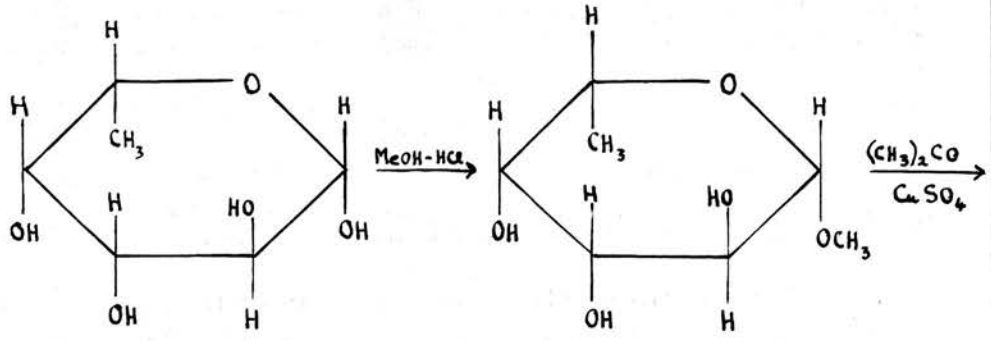
The above syrup was dissolved in 50 ml. of dry acetone and to this was added 5 g. of anhydrous copper sulphate, and five drops of acetaldehyde. The mixture was shaken for 2-3 days, the solution filtered and the residue extracted several times with acetone. After evaporating filtrate and extracts in presence of a little barium carbonate, the residue was extracted with water and again evaporated in presence of barium carbonate. Finally, the residue was extracted with alcohol and the solution evaporated, giving the 3:4-isopropylidene derivative.

Found:	OCH_3, 28.2; $(CH_3)_2CO$, 26.9%
Calc. for $C_{11}H_{10}O_5$	: OCH_3, 28.0; $(CH_3)_2CO$, 26.2%

This derivative was dissolved in methyl iodide (25 ml.) and silver oxide (3 g.) added in ½ g. portions every half hour, the solution being kept at 40° on a water-bath. After refluxing overnight, the solution was filtered, the residue extracted and filtrates evaporated. The process was repeated twice, after which the resulting syrup was transferred to a small flask and distilled in a high vacuum (90-100°/0.04 mm.). Yield 37 mg. $n_D^{16°}$ 1.4555.

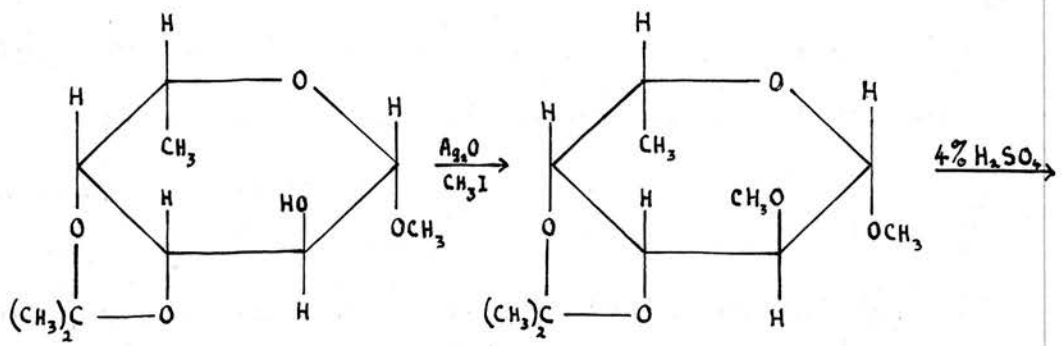
This compound was then hydrolysed with 10 ml. of 4% sulphuric acid at 100° for 6 hours, and the solution/

solution worked up as before. On evaporation, crystalline 2-methyl L-fucose was obtained, m.p. 150-2°.



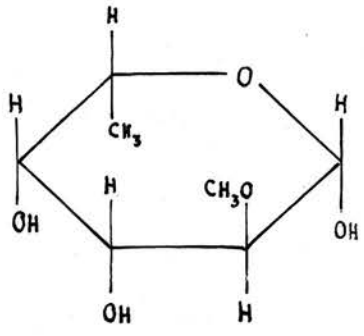
L-FUCOSE

METHYL - L- FUCOPYRANOSIDE



METHYL - 3:4 - ISOPROPYLIDENE - L-FUCOPYRANOSIDE

1:2 - DIMETHYL 3:4 - ISOPROPYLIDENE - L- FUCOPYRANOSIDE



2-METHYL L-FUCOSE

B. Extraction of 3-methyl D-fucose (digitalose) from isoemicymarin. (15).

The cardiac glycoside emicymarin is a much better source of digitalose than isoemicymarin, but unfortunately no samples of this compound were obtainable.

Isoemicymarin (0.1g.) obtained through the kindness of Dr. S. Smith of the Wellcome Chemical Research Laboratories, Beckenham, was dissolved in 50% alcohol (50 ml.) containing 5% of hydrochloric acid, and hydrolysed for 4 hours on a boiling water-bath. The solution was then extracted several times with chloroform (60-70 ml.) and the aqueous fraction neutralised with silver carbonate. After filtration and extraction of the silver residues with hot water, the solution was saturated with hydrogen sulphide and the silver sulphide thus precipitated removed by filtration. The filtrate was evaporated to dryness, when a syrup (20 mg.) was obtained. A further quantity was hydrolysed using 50% alcohol containing normal sulphuric acid, neutralising with barium carbonate, but no better yield was obtained. Part of the syrup was kept for use as a chromatogram standard (see pages 112 and 114).

Formation of digitalonolactone.

The remainder of the syrup (54 mg.) was dissolved in the minimum of water and an excess of bromine was added. After standing for 2-3 days (till a portion of the solution after aeration was non-reducing) the bromine was removed by aeration and the solution treated in the usual manner. On evaporating the final solution of the lactone to dryness and heating to 100°, the solution crystallised, giving needle-shaped crystals, m.p. 136-140°.

Rotation of digitalonolactone.

The lactone (44mg.) was dissolved in water (5 ml.) and the rotation followed.

$[\alpha]_D^{13°}$ -22.0° (0 hours); -24.3° (1 hour); -26.8° (3 hours); -30.9° (5 hours); -34.7° (9 hours); -38.2° (12 hours); -44.8° (17 hours); -53.2° (24 hours); -62.8° (36 hours); -68.4° (42 hours); -70.1° (60 hours, constant).

Comparison of digitalose with Fraction D2.

Since from a comparison of the rotations of the lactones (see page 124), it appeared probable that they were enantiomorphs, it was decided to run specimens of each sugar on a paper chromatogram according to the method described by Partridge (11) using/

using a butanol (40 parts), water (50 parts), and alcohol (10 parts) solvent. Fucose was used as a standard.

1st Paper (28 hrs.)

		R_F ratios
Fucose	8.0 cm.	1
2-methyl fucose	17.0 cm.	2.13
Digitalose	14.3 cm.	1.79

2nd Paper (22 hrs.)

Fucose	6.5 cm.	1
2-methyl fucose	13.8 cm.	2.12
Fraction D2	11.6 cm.	1.78

3rd Paper (24 hrs.)

Fucose	7.8 cm.	1
Digitalose	13.8 cm.	1.77
Fraction D2	13.8 cm.	1.77

Attempted preparation of the acetone compound of Fraction D2.

To the syrup (0.1 g.) was added dry acetone (50 ml.), anhydrous copper sulphate (5 g.) and three drops of acetaldehyde. The solution was shaken for 6 days, filtered, the residue extracted with acetone (200 ml.), and the resulting extracts evaporated in presence of a little barium carbonate. After extraction/

extraction of the residue with water, the filtrate was again evaporated in presence of barium carbonate. Finally, the residue was extracted with alcohol and the extracts evaporated to give a syrup.
Found: $(CH_3)_2CO$, 1.7%.

Examination of Fraction E. (OCH_3, 28.6%)

The syrup (0.39 g.) was hydrolysed with 0.1 N sulphuric acid (50 ml.) at 100° till a constant rotation was obtained $[\alpha]_D^{14°}$ -27.6° ⟶ -15.1°. After neutralisation a syrup (0.35 g.) was obtained.
Found: OCH_3, 17.8%.

A periodic acid oxidation (5) on the syrup gave an acetaldehyde percentage corresponding to 34.5% monomethyl fucose. (See Discussion, page 137).

Formation of a lactone from hydrolysed Fraction E.

The syrup (0.1 g.) was oxidised with bromine till a portion of solution after aeration was non-reducing. After removal of bromine, neutralisation with silver carbonate, and removal of silver with hydrogen sulphide, the solution was transferred to a small flask and distilled (180°/0.04 mm.)
Yield 77 mg. $n_D^{15°}$ 1.4750.
Found: OCH_3, 18.1%
Calc. for a monomethyl fuconolactone: OCH_3, 17.6%
($C_7H_{12}O_5$)

Rotation of lactone.

The lactone (71 mg) was dissolved in water (10 ml.) and the rotation followed.

$[\alpha]_D^{14°}$ +20.2° (20 mins.); +20.2° (30 mins.); +21.8° (1 hour); +25.6° (2 hours); +28.4° (3 hours); +29.2° (4 hours); +33.6° (6 hours); +44.8° (12 hours); +49.7° (18 hours); +52.1° (21 hours); +59.4° (28 hours); +66.8° (36 hours); +74.4° (42 hours, constant).

It thus appeared that the lactone was a γ-lactone, indicating that C_4 had been unsubstituted in the methylated sugar.

Titration of the lactone.

Titration of 2 ml. of the above rotation solution showed that the acid solution required 2.66 ml. of 0.02505 N sodium hydroxide. (Calc.: 2.96 ml.).

Amide formation.

To the lactone (70 mg.) was added methanolic ammonia (2 ml.) and the solution was allowed to stand at 0° for 48 hours. On evaporation at 15°/15 mm. a syrup was obtained which after several weeks partially crystallised, giving a product m.p. 176-180°.

$[\alpha]_D^{15°}$ +16.4° (c, 0.58 in water).

Found: OCH_3, 16.4 %

Calc. for $C_7H_{15}O_5N$: OCH_3, 16.1 %

Weerman test on amide.

The test was carried out as already described on page 123.

42.57 mg. of amide gave 20.10 mg. of hydrazodicarbonamide, m.p. 256° (decomp.). In another experiment, the amide (47.3 mg.) gave 15.4 mg. of hydrazodicarbonamide, m.p. 254°. A control experiment with gluconamide (92.79 mg.) gave 39.21 mg. of precipitate, m.p. 256° (decomp.).

Examination of Fraction F. (OCH_3, 30.9%)

A small portion of this fraction after hydrolysis with 0.1 N sulphuric acid was examined by means of a paper chromatogram and found to be identical with Fractions D2 and E. It thus contained 3-methyl methyl-L-fucosides.

Examination of Fraction G. (OCH_3, 16.4%)

This fraction partially crystallised and the crystals obtained (0.18 g.) had the following constants, after recrystallisation from ethyl acetate:-
m.p. 154-155°; mixed m.p. with authentic α-methyl-L-fucoside 155°.

$[\alpha]_D^{15°}$ -194° (c, 1.10 in water).

Found: OCH_3, 17.0%

Calc. for $C_7H_{14}O_5$: OCH_3, 17.4%

It thus appeared certain that the crystals were α-methyl-L-fucoside.

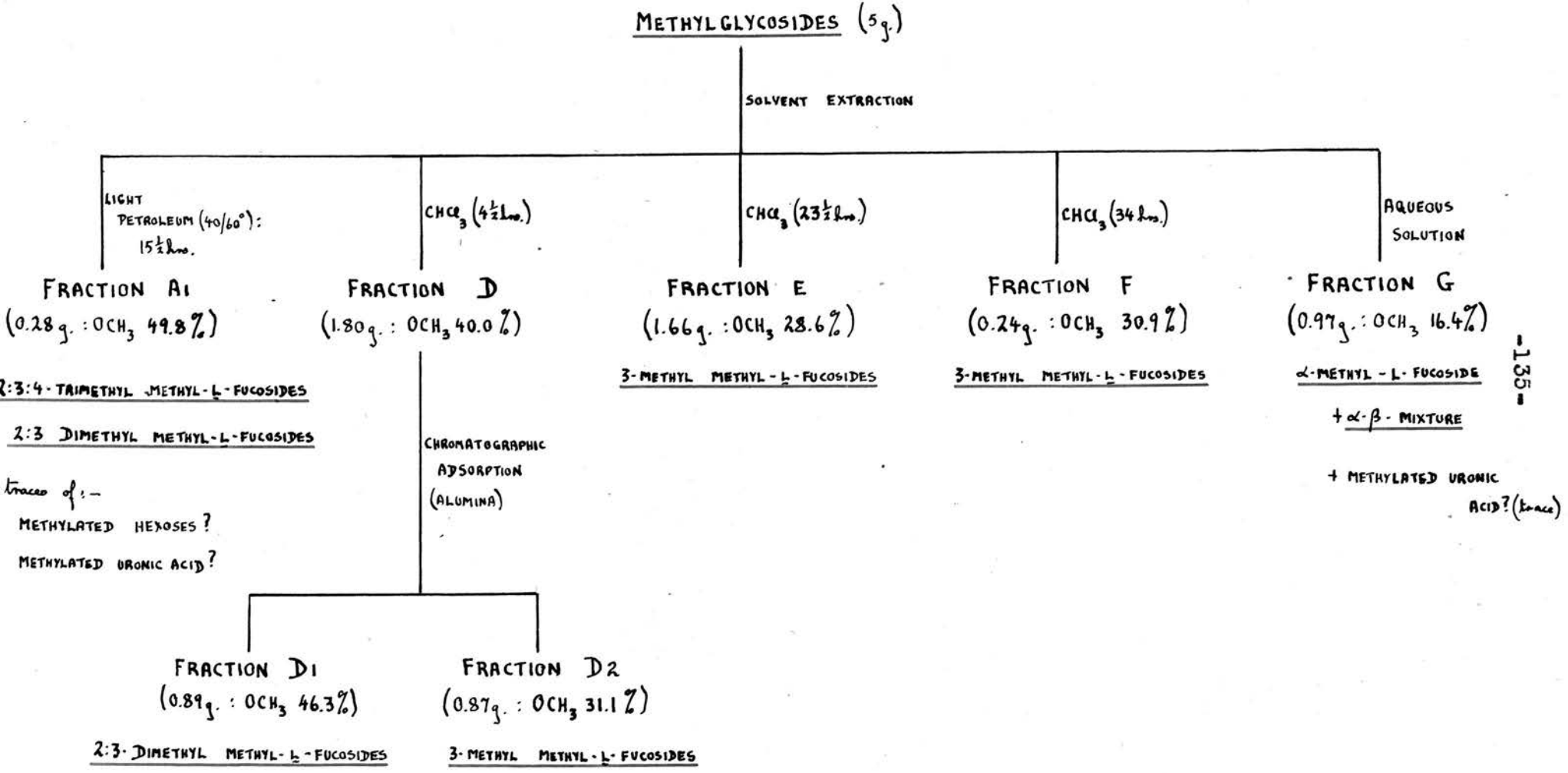
Examination by paper chromatography showed the fraction, after hydrolysis with 0.1 N sulphuric acid, to consist of fucose with a trace of what was possibly a uronic acid. A fucose estimation by periodic acid oxidation gave 90.3% fucose.

Examination of Fraction A1 (OCH_3, 49.8%)

The fraction was a highly mobile syrup, the methoxyl content of which (49.8%) suggested that it was composed of highly methylated derivations. After hydrolysis with 0.1 N sulphuric acid for 8 hours, the syrup was examined by means of a paper chromatogram.

Standards		Hydrolysed A1
		3.0 cm.
Fucose	8.4 cm.	
2-methyl fucose	14.4	
2:3-dimethyl fucose	23.1	23.0
2:3:4-trimethyl fucose	28.2	28.0
		30.5 (trace)
		32.0 (trace)

From the above results it was observed that fucose, and 2- and 3-methyl fucose were all definitely absent. The dimethyl fucose found in Fractions D1 and II, i.e. 2:3-dimethyl-L-fucose, and 2:3:4-trimethyl fucose appeared to be present in quantity, together with traces of one or more highly methylated hexoses or pentoses. There was also a possible indication of the presence of a small amount of a methylated uronic acid.


METHYL GLYCOSIDES (5g.)
SOLVENT EXTRACTION
LIGHT PETROLEUM (40/60°): 15½ hrs.
CHCl3 (4½ hrs.)
CHCl3 (23½ hrs.)
CHCl3 (34 hrs.)
AQUEOUS SOLUTION
FRACTION A1
(0.28g. : OCH3 49.8%)
2:3:4 - TRIMETHYL METHYL - L - FUCOSIDES
2:3 DIMETHYL METHYL - L - FUCOSIDES
traces of :-
METHYLATED HEXOSES ?
METHYLATED URONIC ACID ?
FRACTION D
(1.80g. : OCH3 40.0%)
CHROMATOGRAPHIC ADSORPTION (ALUMINA)
FRACTION D1
(0.89g. : OCH3 46.3%)
2:3 - DIMETHYL METHYL - L - FUCOSIDES
FRACTION D2
(0.87g. : OCH3 31.1%)
3 - METHYL METHYL - L - FUCOSIDES
FRACTION E
(1.66g. : OCH3 28.6%)
3 - METHYL METHYL - L - FUCOSIDES
FRACTION F
(0.24g. : OCH3 30.9%)
3 - METHYL METHYL - L - FUCOSIDES
FRACTION G
(0.97g. : OCH3 16.4%)
α - METHYL - L - FUCOSIDE
+ α - β - MIXTURE
+ METHYLATED URONIC ACID? (trace)

DISCUSSION.

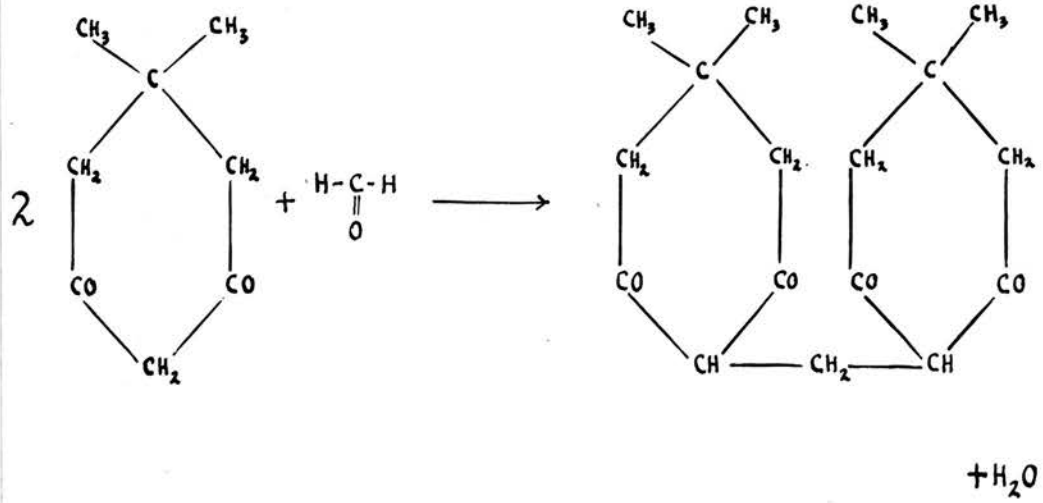
After a preliminary treatment with pyridine according to the method employed by Pacsu and Mullen (17) for starch, fucoidin D on acetylation gave a good yield of an acetate, the acetyl value of which (18.0%) appeared to indicate that there existed in the polysaccharide approximately one free hydroxyl group for each fucose residue.

Methylation of the polysaccharide with dimethyl sulphate and sodium hydroxide proved to be rather a lengthy process, as the methylation had to be carried out in water, and the resulting sodium sulphate removed by dialysis. It was found that methylation of the acetate (which gave a product OCH_3, 6.39%) was no more effective than direct methylation of the polysaccharide (OCH_3, 7.49%), so in all experiments the direct method was used. After four methylations a product was obtained, the methoxyl content of which could not be raised by further treatment. An attempt to form the acetate of this methylated compound resulted in a product with an acetyl value of 1% which appeared to confirm that the polysaccharide was fully methylated. A further methylation carried out in the cold did not raise the methoxyl content appreciably. The final product had a methoxyl content of between 15.5% and 15.8%, though the intermediate values/

values differed slightly in different experiments.

As in the case of the unmethylated polysaccharide, oxidation by periodic acid of the hydrolysed fractions was used as a method of estimating fucose. Theoretically, the method should also have been applicable to methylated fucoses provided positions 4 and 5 were occupied by hydroxyl groups (the sugar is assumed to react with periodic acid in the open chain form). In practice it was found, however, that even in the case of relatively pure fractions of methylated fucoses, where C_4 and C_5 appeared by other evidence to be free, the yield of acetaldehyde was not quantitative, and in most cases represented about one-third (32-34%) of the theoretical yield.

Some interesting observations which may have some bearing on this anomaly have recently been published by Bell (16). After finding that 2:3-dimethyl glucose could easily be determined by periodate oxidation of the sugar and estimation of the formaldehyde produced, by formation of a formaldehyde-dimedone complex:-



$+H_2O$

Bell studied the possible extension of the procedure to other partially methylated aldohexoses possessing a free primary hydroxyl group. It had already been found by Jeanloz (17) that 2:3:4-trimethyl glucose oxidised in a bicarbonate buffer could not be made to yield more than a fraction of the formaldehyde expected. Using a phosphate buffer, Bell found that similar results were obtained with 2:4- and 3:4-dimethyl galactoses, 3:4-dimethyl mannose and 3-methyl glucose, while on the other hand, 2-methyl glucose and 2:3-dimethyl galactose yielded the theoretical quantities of formaldehyde-dimedone at the same rate as 2:3-dimethyl glucose.

The reason for these anomalous oxidations is not yet clear, but work in progress in Bell's laboratory has indicated that the action of periodate on partially substituted sugars and on free sugars can be modified by the presence of phosphate ion. Unpublished work of his with A.T. Johns and Miss A. Palmer has so far shown that at pH 7.5, in a phosphate buffer secondary oxidations result in the production of carbon dioxide and of steam-volatile acids (largely formic) where such would be expected normally to be formed.

While in the estimations of fucose carried out during the course of this research no phosphate buffer was used, although the reaction was carried out in the presence of excess sodium bicarbonate, it is extremely probable/

probable that a similar explanation holds. The method, even though it could not be applied quantitatively, proved useful as a qualitative test, for if position C_4 carried no free hydroxyl group, then no reaction at all would have occurred with periodic acid.

The methylated polysaccharide $[\alpha]_D^{15°}$ -106.6° (c, 1.3 in water) was found to have similar properties to the original fucoidin. The ash as sulphate was 21.17% and when analysed gave calcium 24.13; magnesium 0.97; sodium 3.80; potassium 0.24; sulphate 69.67%. As before, the total sulphate (25.93%) was approximately double the sulphate in the ash (14.75%), indicating that the Haas ethereal sulphate formula still applied to the methylated compound. It is rather surprising that little or no calcium or magnesium was replaced by sodium during methylation. The sulphate content of the methylated polysaccharide (25.9%) shows that little or no sulphate has been split off, for the total sulphate content of the original fucoidin was 32.8%, which in the methylated polysaccharide, allowing for a methoxyl of 15.5% would give a theoretical sulphate content of 27.7%.

Large quantities of the polysaccharide were not easily obtained, chiefly owing to the difficulty involved in dealing with large volumes of extract, and their subsequent treatment, e.g. dialysis. This, together with the fact that the yields on methylation

were/

were rather poor, resulted in the hydrolysis and fractionation of the polysaccharide being carried out on smaller quantities than was perhaps desirable.

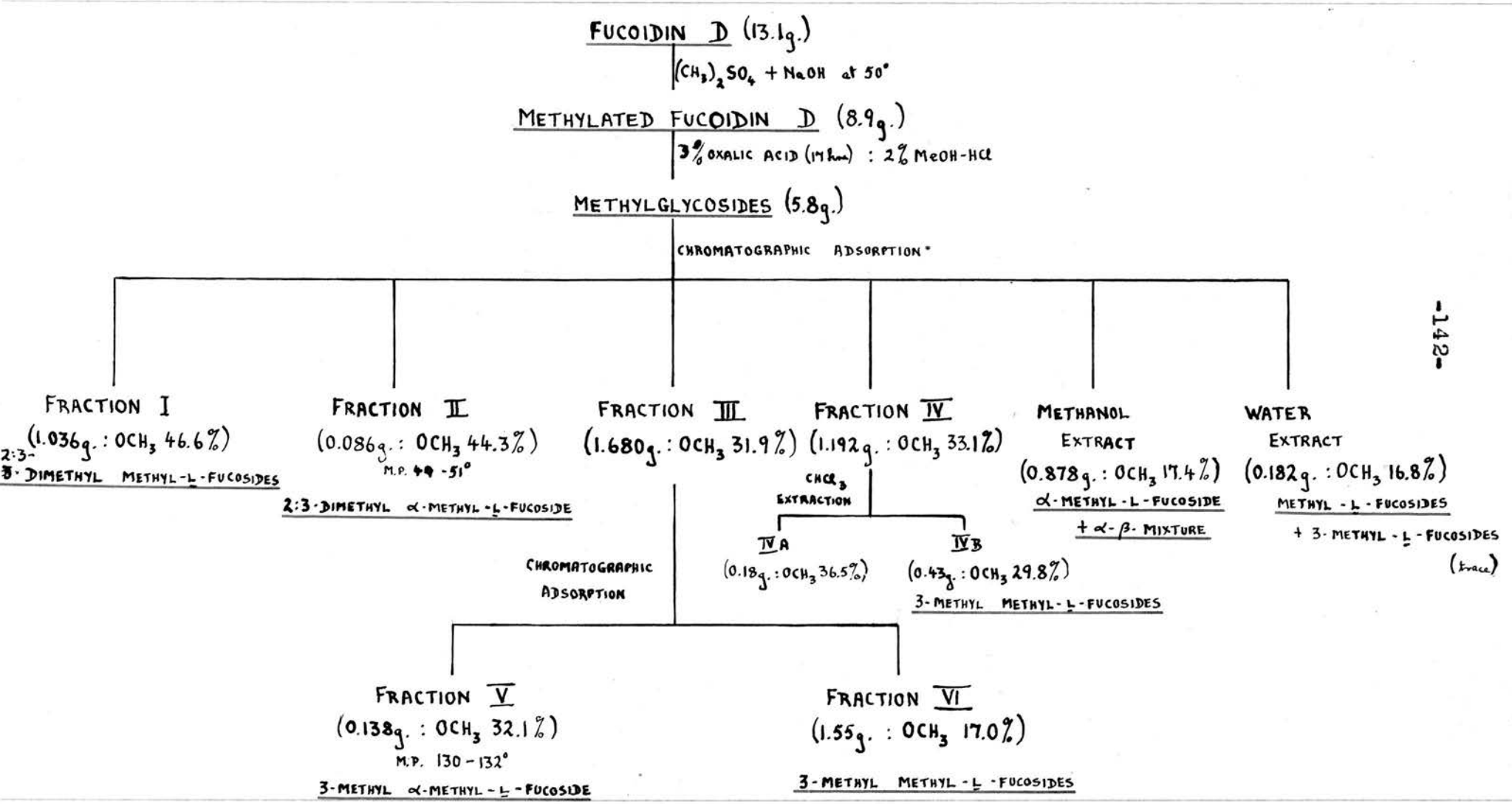
In a preliminary experiment after hydrolysis of the methylated polysaccharide with 3% oxalic acid, the glycosides of the sugars so obtained, were formed and distilled. A portion of the glycosides was hydrolysed with 0.5 N sulphuric acid till a constant rotation was observed, whereupon an attempt was made to form an osazone. The resulting product appeared on analysis to be a monomethyl osazone of fucose, which suggested that in the hydrolysed methylated polysaccharide, at least some of the fucose residues had position C_2 free, i.e. occupied by a hydroxyl group, indicating that the position had been used in some type of linkage either to a sulphate group or another fucose residue.

The remainder of the glycosides were treated with anhydrous copper sulphate and acetone (page 98) in an endeavour to form acetone compounds of the methyl-fucosides. It was considered that it might be possible to fractionate the hydrolysed polysaccharide in this way. α -methylfucopyranoside easily forms a 3:4-isopropylidene compound (page 126) and therefore if the two adjacent 3:4 hydroxyl groups were free in any of the methylated fractions an acetone compound would be formed, and since such compounds have a very

low/

low boiling-point, such a fraction could be collected first on distillation. On distilling in a high vacuum after the above treatment three fractions were obtained, the acetone content of which did not appear to indicate the formation of any appreciable quantity of acetone compounds. This suggested that in any monomethyl fucose residues present position C_3 was occupied by some substituent.

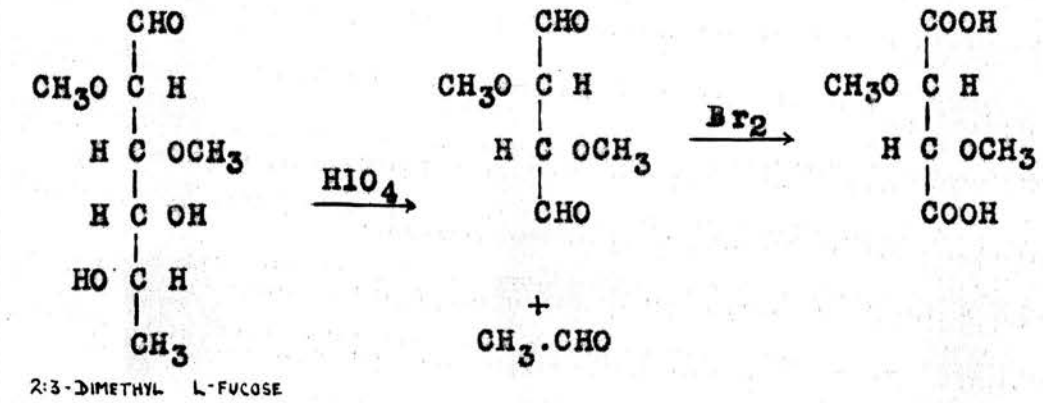
Since no separation could be effected in this way, the method of chromatographic adsorption adapted from Jones (10) was next employed (page 101). By developing with selected solvents, e.g. chloroform and petroleum-ether mixed in varying proportions, it is possible by this method to separate the more fully methylated methylglycosides, which are not so strongly adsorbed on the column of alumina and are thus more easily washed through, from those not so fully methylated. Using this method, a series of fractions was obtained, which, after combining consecutive portions with similar refractive indices, may be summarised as shown overleaf.


FUCOIDIN D (13.1g.)
$(CH_3)_2SO_4$ + NaOH at 50°
METHYLATED FUCOIDIN D (8.9g.)
3% OXALIC ACID (14 hrs.) : 2% MeOH-HCl
METHYLGLYCOSIDES (5.8g.)
CHROMATOGRAPHIC ADSORPTION*
FRACTION I
(1.036g. : OCH_3 46.6%)
2:3-
3-DIMETHYL METHYL-L-FUCOSIDES
FRACTION II
(0.086g. : OCH_3 44.3%)
M.P. 49-51°
2:3-DIMETHYL α-METHYL-L-FUCOSIDE
FRACTION III
(1.680g. : OCH_3 31.9%)
FRACTION IV
(1.192g. : OCH_3 33.1%)
METHANOL EXTRACT
(0.878g. : OCH_3 17.4%)
α-METHYL-L-FUCOSIDE
+ α-β- MIXTURE
WATER EXTRACT
(0.182g. : OCH_3 16.8%)
METHYL-L-FUCOSIDES
+ 3-METHYL-L-FUCOSIDES
(trace)
$CHCl_3$ EXTRACTION
IV A
(0.18g. : OCH_3 36.5%)
IV B
(0.43g. : OCH_3 29.8%)
3-METHYL METHYL-L-FUCOSIDES
CHROMATOGRAPHIC ADSORPTION
FRACTION V
(0.138g. : OCH_3 32.1%)
M.P. 130-132°
3-METHYL α-METHYL-L-FUCOSIDE
FRACTION VI
(1.55g. : OCH_3 17.0%)
3-METHYL METHYL-L-FUCOSIDES

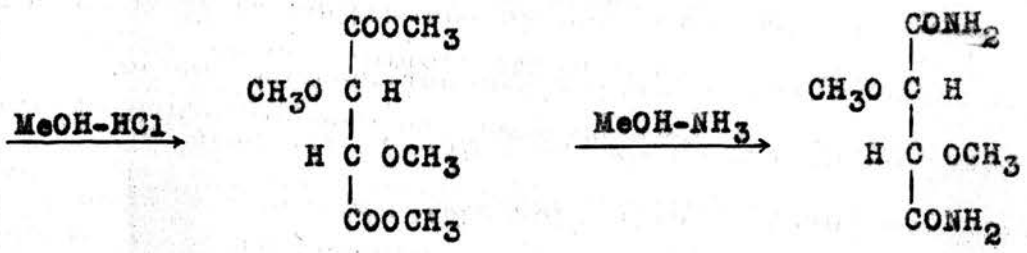
Of these fractions, II was crystalline while III was partly crystalline. Fraction II being available in such small quantity could not be examined in great detail but the analytical figures, taken in conjunction with the fact that it appeared amongst the dimethyl fraction, point to it being a dimethyl methylfucoside, while its high negative rotation suggested an α-methyl-L-fucoside (page 109).

Fraction I (page 105) which appeared to be homogeneous was shown to be an L-fucose derivative by the complete methylation of part of it to give 2:3:4-trimethyl α-methyl-L-fucoside, identified by comparison with an authentic specimen. Valuable information as to the positions occupied by methoxyl groups was gained by the oxidation of part of this fraction with periodic acid, followed by oxidation with bromine water and formation of the ester and amide of the acid so produced. The resulting product appeared to be D(-)-dimethoxysuccinamide on comparison with an authentic specimen. This at once suggests that the methoxyl groups in the fucose molecule are in the C_2 and C_3 positions, as only in these positions could D(-)-dimethoxysuccinamide be formed.

Briefly summarised the reaction would occur as follows:-



2:3-DIMETHYL L-FUCOSE



D(-)-DIMETHOXYSUCCINAMIDE

From a study of the formula it will be seen that of the other possibilities, neither 2:4-dimethyl nor 3:4-dimethyl fucose could react with periodic acid in this way. Thus it would appear that 2:3-dimethyl L-fucose was present. The yields from this reaction were poor, however, so the presence of a proportion of either of the other dimethyl fucoses could not be excluded.

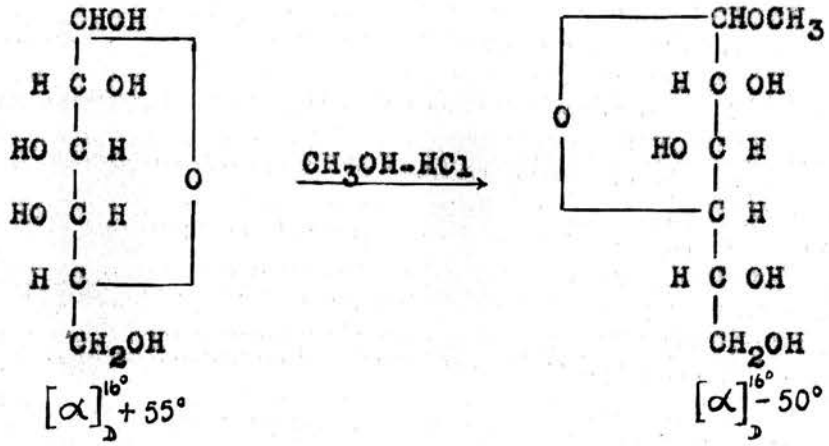
Fraction III (page 109), although purified by passing a solution of it through a column of alumina, did not yield any more crystals than were originally obtained, so it was presumed that the rest of the syrup was a mixture of α- and β-methylglycosides since both syrup and crystals appeared on hydrolysis and examination by the paper chromatogram method by comparison/

comparison with digitalose (3-methyl D-fucose) and 2-methyl L-fucose, to be the same sugar, namely 3-methyl L-fucose. Analysis of the crystals gave figures closely resembling those for a monomethyl fucoside, while the high negative rotation suggested the α-form. Thus the compound, m.p. 130-132°; $[\alpha]_D^{14°}$ -173.1° (c, 0.4 in water) is to be described as 3-methyl α-methyl-L-fucoside.

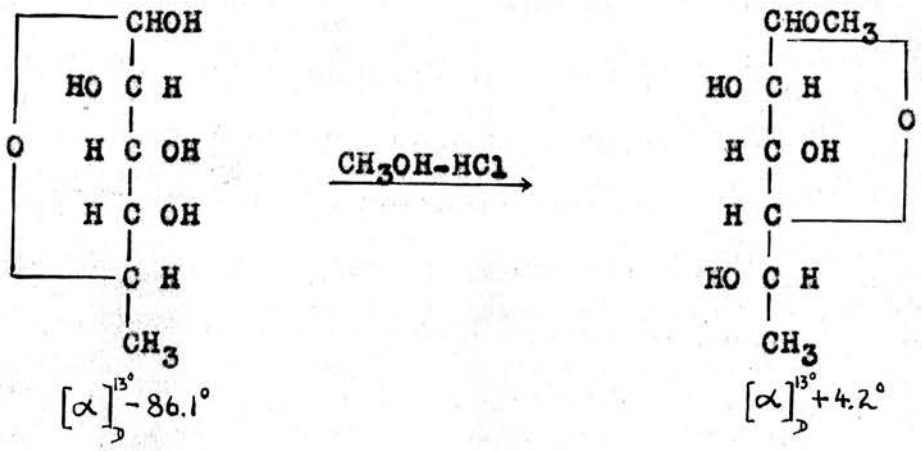
Fraction IV (OCH_3, 33.1% page 110) appeared from its methoxyl content to be composed of a mixture of methylated sugars, and an attempt was made to extract the more highly methylated fraction by shaking with chloroform. The results appeared to indicate that some separation had been achieved though not a very effective one.

Both fractions IVa (OCH_3, 36.5%) and IVb (OCH_3, 29.8%) were proved to be methylated fucoses by complete methylation to give 2:3:4-trimethyl α-methyl-L-fucoside. Further evidence that C_4 was free in IVb (composed mainly of monomethyl fucose) was provided by indications of furanoside formation, when the free sugar was allowed to stand with 1% methanolic hydrogen chloride (page 112). This test has been used previously in the galactose series (6); for example, galactose and 2:6-dimethyl D-galactose on treatment with dilute methanolic hydrogen chloride in the cold, suffered a change/

change in rotation from positive to negative:-



owing to the formation of the furanoside with the ring on the left in the projection formula. For L-fucose (6-desoxy-L-galactose) a change in the opposite sense may be interpreted in the same way.



In addition, the solution from this experiment yielded an osazone which still contained a methoxyl group, indicating that C_2 also carried a free hydroxyl group.

Fraction VI (OCH_3, 31.9%; page 114), the syrup from the crystals, Fraction V, (page 113), appeared from paper chromatographic examination to be identical with Fraction V and thus consisted of 3-methyl methyl-L-fucosides.

The products of the methanol and water extractions appeared on examination to be mixtures, with unsubstituted methylfucosides predominating, and a sample of crystalline α-methyl-L-fucoside was obtained from the alcohol extract.

Considering the proportions of fucose residues in various states of methylation, the 2:3-dimethyl methylfucoside appears to be present to the extent of ca. 22% while the 3-methyl methylfucoside comprises at least 57% of the syrup. The remainder (21%), though mostly unsubstituted methylfucosides cannot be considered as being composed entirely of such, since there is a little 3-methyl methylfucoside and probably a trace of methylated uronic acid also present. There are, however, approximately three monomethyl and one dimethyl fucose residues for every free fucose residue.

A further attempt (page 117) at separating the methylated fractions was made using the technique of solvent extraction as developed by Brown and Jones (12). This method is basically the same as the chromatographic adsorption method, depending on the differing solubilities of the methylated sugars in various solvents. It proved to be more useful in that it eliminated the difficulty of extracting the last trace of syrup from the column, although unfortunately, the separation was not so clear-cut and a chromatographic separation had also to be carried out on one fraction. The fractions may be summarised as follows:-

Fraction A1 (page 134) contained several methylated sugars, but the fraction was so small that no attempt at separation was made. Comparison with standards on a paper chromatogram indicated that both 2:3-dimethyl fucose and 2:3:4-trimethyl fucose were present, together with some highly methylated hexoses or pentoses and what appeared to be a trace of methylated uronic acid.

Fraction D (page 118) as obtained from the solvent extraction of the sugars was obviously a mixture, and was separated by means of a column of alumina into two fractions, D1 and D2, with methoxyl contents corresponding to di- and mono-methyl methylfucosides respectively. The methoxyl content of D1 (46.3%) was a little higher than the theoretical value for a dimethyl methylfucoside (45.1%) which pointed to the presence of a trace of trimethyl methylfucoside, a fact which was confirmed by a paper chromatogram examination, when a faint spot corresponding to trimethyl fucose was observed. As was the case with the chromatographic adsorption separation of the glycosides (page 101), this fraction (D1) was obtained in a partly crystalline state, but to avoid any loss, no attempt was made at separation, the fraction being considered as a mixture of the dimethyl α- and β-methylfucosides. An attempt to form an anilide was unsuccessful/

unsuccessful, and the dimethyl sugar could not be crystallised. The lactone of the sugar acid did not crystallise, and from a titration with 0.025 N sodium hydroxide (page 122), appeared to be slightly impure. The amide prepared from the lactone (page 122) was, however, crystalline. A Weerman test on this amide gave no precipitate, so it appeared definite that C_2 was occupied by a methoxyl group. This, together with the results obtained from the examination of Fraction I in the previous hydrolysis (page 105) already discussed, can be considered conclusive proof that the fraction was composed of 2:3-dimethyl methyl-L-fucosides.

Fraction D2 (OCH_3, 31.1%) appeared to have position C_4 occupied by a hydroxyl group, as the free sugar showed signs of furanoside formation (page 123; (6)) when allowed to stand with 1% methanolic hydrogen chloride. A crystalline lactone was obtained having a melting-point (132-136°) similar to that of digitalonolactone (136-140°) and a rotation $[\alpha]_D^{14°}$ +75.1° (c, 0.95 in water) which though not quite equal and opposite in sign to that quoted for digitalonolactone $[\alpha]_D^{17°}$ -83° (c, 3.23 in water) by Lamb and Smith (15) was of the same order. Furthermore, the courses of hydrolysis of the two lactones in water were closely similar (pages 124 and 129). When the free sugar from D2 and digitalose were/

were compared on a paper chromatogram they appeared to be identical. It is known that the D- and L- forms of any sugar travel at the same rate (18) so this was strong evidence as to the identity of the sugar. Further evidence that C_2 was not occupied by a methoxyl group was provided by the failure to form an acetone compound of the Fraction D2 (page 130). This would have been readily formed had positions C_3 and C_4 both been occupied by hydroxyl groups.

Fraction E (OCH_3, 28.6%) appeared from chromatograms to be identical with Fraction D2 though a slight trace of free fucose was observed. The lactone also had a rotation ($[\alpha]_D^{14°}$ +20.2° ⟶ +74.4° in 42 hours) similar to that of the D2 lactone, while the amide which was obtained partly crystalline (page 132) gave a precipitate of hydrazodicarbonamide on carrying out a Weerman test. The amount of free fucose, as observed on the chromatogram, could not (by conversion to the corresponding amide) have been sufficient to account for the yield of hydrazodicarbonamide obtained. Titration of the lactone indicated that the fraction was only 90% pure and it was presumably this impurity which prevented crystallisation of the lactone.

Fraction F (OCH_3, 30.9%) was found on examination by means of a paper chromatogram (page 133) and comparison with standards - digitalose, fucose

and/

and 2-methyl L-fucose to be composed of 3-methyl methyl-L-fucosides.

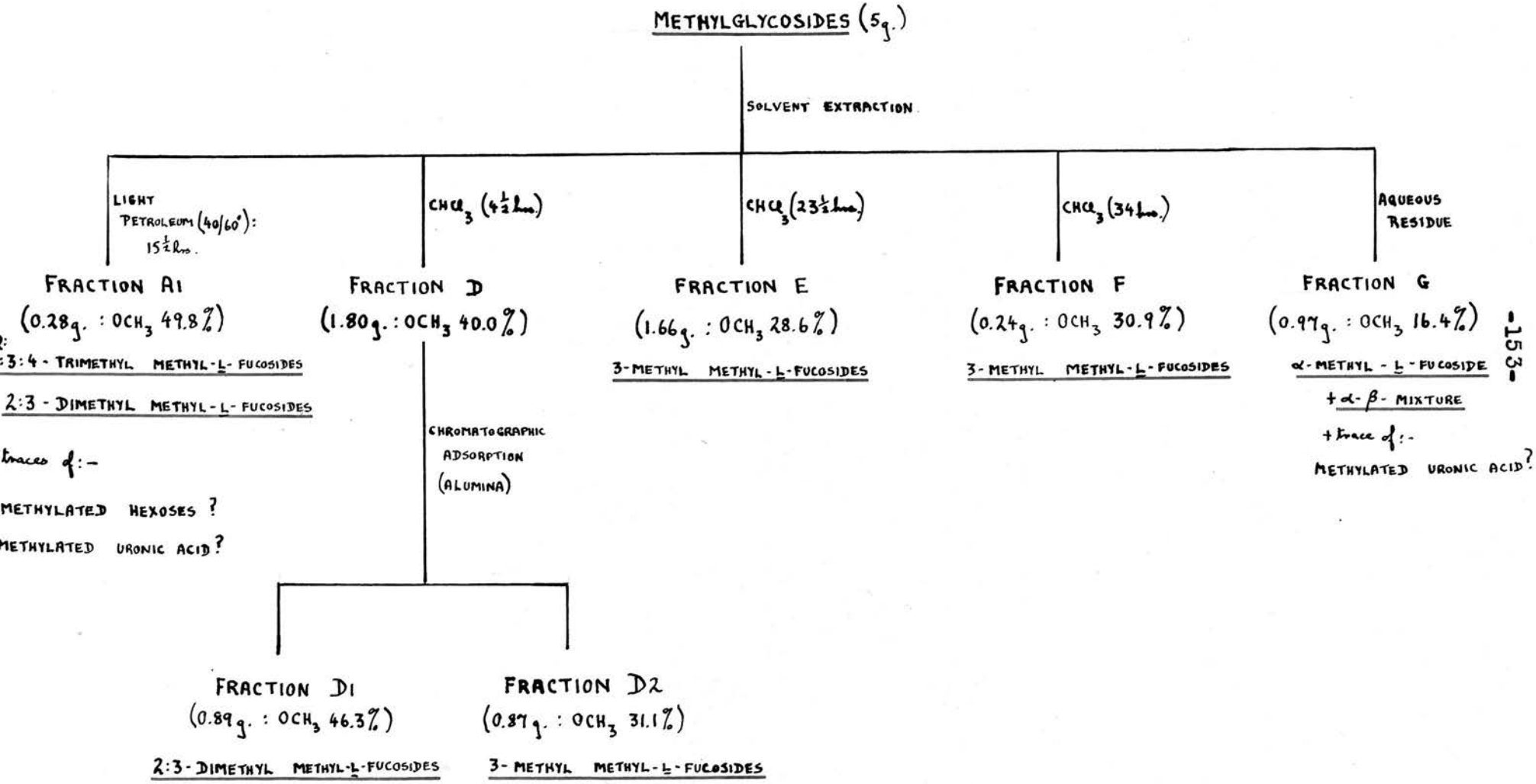
Fraction G (OCH_3, 16.4%) the aqueous residue, was found to consist almost entirely of free methylfucosides with a small quantity of a uronic acid present.

Summarising, it appears that, as was found in the investigations by separation in an alumina column, the main fraction is 3-methyl methyl-L-fucoside, while approximately equal quantities of 2:3-dimethyl methyl-L-fucosides and methyl-L-fucosides are present. There also appears evidence for the presence of a small quantity of trimethyl-L-fucoside. The relative proportions are approximately as follows:-

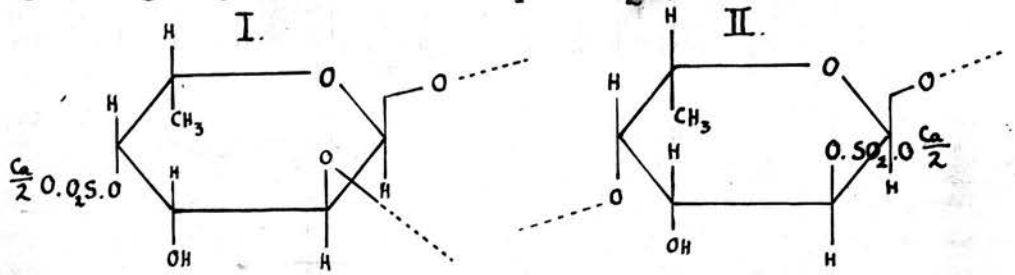
3-methyl methyl-L-fucosides (56%); 2:3-dimethyl methyl-L-fucosides (18%); methyl-L-fucosides (18%).

Thus, as in the case of the chromatographic adsorption separation, the methylated methylfucosides are present in the proportions - 3-methyl methyl-L-fucosides (3 parts), 2:3-dimethyl methyl-L-fucosides (1 part) and methyl-L-fucosides (1 part). It thus appears that in the methylated polysaccharide the basic building unit is 3-methyl L-fucose.

A flow-sheet summarising the fractions is given on page 153.


METHYLGLYCOSIDES (5g.)
SOLVENT EXTRACTION
LIGHT PETROLEUM (40/60°): 15½ hrs.
CHCl3 (4½ hrs.)
CHCl3 (23½ hrs.)
CHCl3 (34 hrs.)
AQUEOUS RESIDUE
FRACTION A1
(0.28g. : OCH3 49.8%)
3:4 - TRIMETHYL METHYL-L- FUCOSIDES
2:3 - DIMETHYL METHYL-L- FUCOSIDES
traces of:-
METHYLATED HEXOSES ?
METHYLATED URONIC ACID?
FRACTION D
(1.80g. : OCH3 40.0%)
CHROMATOGRAPHIC ADSORPTION (ALUMINA)
FRACTION E
(1.66g. : OCH3 28.6%)
3-METHYL METHYL-L-FUCOSIDES
FRACTION F
(0.24g. : OCH3 30.9%)
3-METHYL METHYL-L-FUCOSIDES
FRACTION G
(0.97g. : OCH3 16.4%)
α-METHYL-L-FUCOSIDE
+α-β- MIXTURE
+trace of:-
METHYLATED URONIC ACID?
FRACTION D1
(0.89g. : OCH3 46.3%)
2:3-DIMETHYL METHYL-L-FUCOSIDES
FRACTION D2
(0.87g. : OCH3 31.1%)
3-METHYL METHYL-L-FUCOSIDES

Considering the structure of the polysaccharide, the question of the position of the sulphate group arises. Alkaline hydrolysis of the original polysaccharide D showed that the sulphate group strongly resisted hydrolysis (page 66), but, while in the case of a hexose unit this can be taken as an indication that the sulphate group is not in a position whereby a 3:6 anhydro ring could be formed on hydrolysis, in the case of a methylpentose this cannot hold. If it is assumed that in the methylated polysaccharide 3-methyl L-fucose is the principal unit in the chain, and that the fucose residues are pyranose, the ease of hydrolysis not being comparable with that of galactocarolose (21), which contains galactofuranose units, then obviously the sulphate group could be on C_4 or C_2, thus:-



It would be expected that in II, with the sulphate group <u>trans</u> to the free hydroxyl group on C_3, the sulphate residue would be eliminated readily with alkali, with the formation of an ethylene oxide ring (19). In I, on the other hand, with the sulphate group and the hydroxyl group on C_3 <u>cis</u>, ready elimination of sulphate with alkali would not be expected, by analogy with methanesulphonic esters with adjacent <u>cis</u> hydroxyl groups (20).

The linkages appear, from the high negative rotation/

rotation of fucoidin and the fact that the principal unit is L-fucose to be α- in character.

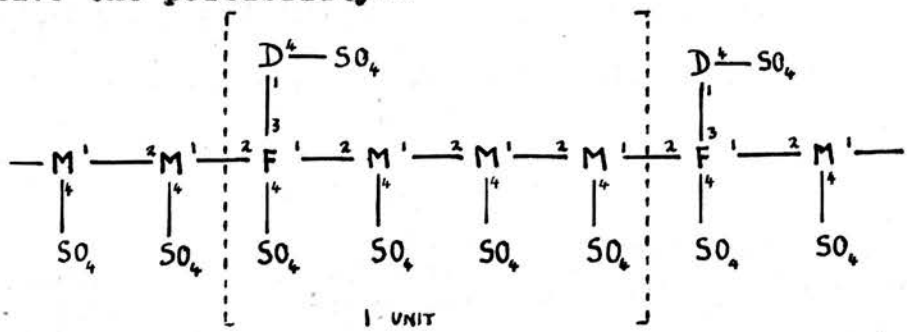
While it is perhaps premature to advance possibilities for the structure of the fucose portion of the molecule, especially when even the character of the remainder is undecided, the above results do appear to permit of one relatively simple suggestion. Considering the methylated polysaccharide, then if

M 3-methyl fucose

D 2:3-dimethyl fucose

and F fucose

we have the possibility:-



This formula, the simplest which can be postulated on the above proportions, allows one sulphate group on each fucose residue, and in the above case it is assumed all are on position C_4.

The fact that a dimethyl methyl-fucoside has been isolated could mean that sulphate residues had been lost by hydrolysis during methylation, but the proportion of dimethyl methylfucosides is so high and the loss of sulphate on methylation so small (1.8%) that this seems highly unlikely.

Furthermore/

Furthermore, if the deductions based on the difficulty of removing the sulphate group by alkali are correct, and thus I is the most likely arrangement for those fucose residues carrying a sulphate group, then the dimethyl fucose formed on hydrolysis would be the 3:4 isomer and not the 2:3-, and no evidence in that direction was obtained.

It is, therefore, highly probable that the 2:3-dimethyl methylfucoside arises from a terminal branching point in a chain.

The proportion of trimethyl methylfucoside obtained was very small, and may have arisen from the hydrolysis of sulphate from a small amount of the "D" residues.

As regards the unidentified portion of the molecule, no evidence for its presence was found in the methylated products. It thus appears to vanish either on methylation or on hydrolysis with methanolic hydrogen chloride and the experiments on methylated fucoidin have thrown light only on the portion of the polysaccharide made up of fucose units. It is quite impossible to say at present whether the unidentified part of the molecule is composed of impurities or is part of the structural pattern.

It must be again emphasised that the above formula is not put forward as the proposed structure of the fucose portion, but merely as the simplest structure possible with the proportions obtained, and methods such as partial degradation of the polysaccharide will

have/

have to be attempted before a definite structure can be reached. It is more than probable that the molecule is a highly complex one with many branches, both simple and multiple. Even in the case of those polysaccharides, the composition of which has been fully elucidated, it is not possible to write formulae because of the various possible structures which may be written for each of the compounds. More accurate methods for the determination of molecular weights and of end groups are required and it is particularly important to find methods for distinguishing between single and multiple branched structures. At present it is not possible to distinguish between single branching and multiple branching, so it is quite possible that many polysaccharides are considerably more complex than has been postulated.

SUMMARY.

1. The polysaccharide, fucoidin D, was acetylated to give an acetate (CH_3CO, 18.0 %).

2. Methylation of the acetate was found to be no more satisfactory than direct methylation and so the polysaccharide was methylated directly with dimethyl sulphate and sodium hydroxide giving, after four methylations, a product with a methoxyl content of 15.5 - 15.8 %.

3. The methylated polysaccharide $[\alpha]_D^{15°}$ -106.6° (c, 1.3 in water), gave ash as sulphate 21.2 %. Analysis of the ash gave calcium 24.1 % (5.11 % of the polysaccharide); magnesium 0.97 % (0.21 %); potassium 0.24 % (0.05 %); sodium 3.80 % (0.80 %); sulphate 69.7 % (14.75 %). The total sulphate was 25.9 %.

4. The methylated polysaccharide was hydrolysed with 3 % oxalic acid and a monomethyl osazone (m.p. 178-179°) was obtained, probably the osazone of 3-methyl L-fucose.

5. Attempts to condense the methylglycosides, obtained from the hydrolysed methylated polysaccharide, with acetone were unsatisfactory.

6. A separation of the methylglycosides was effected by means of chromatographic adsorption. Fractions were obtained as follows:-

(a) Fraction I (2:3-dimethyl methyl-L-fucosides) on complete methylation gave 2:3:4-trimethyl α-methyl-L-fucoside.

Oxidation with periodic acid and bromine water followed by esterification and formation of the amide gave D(-)-dimethoxysuccinamide.

(b) Fraction II was shown to be a dimethyl methyl-fucoside, presumably 2:3-dimethyl α-methyl-L-fucoside.

(c) Fraction III was refractionated on a column of alumina, giving a crystalline fraction V, shown to be 3-methyl α-methyl-L-fucoside, and a syrup VI which appeared to be a mixture of α- and β-methylglycosides of the same sugar.

(d) Fraction IV was extracted with chloroform to give two fractions, both of which on complete methylation yielded 2:3:4-trimethyl α-methyl-L-fucoside. Fraction IVb was shown to have a free hydroxyl group on C_4 by furanoside formation, and a free hydroxyl group on C_2 by formation of a methyl osazone. Comparison with digitalose on a paper chromatogram indicated that the fraction was composed of 3-methyl methyl-L-fucosides.

(e) The fractions extracted by methanol and by water were shown to be mainly free methylfucosides, and crystalline α-methyl-L-fucoside was isolated.

7. Separation by means of solvent extraction gave the following fractions:-

(a) Fraction A1: a mixture of dimethyl and trimethyl methylfucosides, together with traces of methylated hexoses and possibly a methylated uronic acid.

(b) Fraction D was separated by chromatographic adsorption into:-

D1 which, on examination of the derived lactone and amide, was identified as 2:3-dimethyl methyl-L-fucosides.

D2 identified from its lactone and amide and by comparison with digitalose (3-methyl D-fucose) and digitalonolactone as 3-methyl methyl-L-fucosides.

(c) Fraction E identified from the properties of its lactone and amide, and by comparison with digitalose, as 3-methyl methyl-L-fucosides.

(d) Fraction F identified by chromatographic comparison with digitalose as 3-methyl methyl-L-fucosides.

(e) Fraction G shown to be a mixture of methylfucosides, with traces of methylated hexoses and possibly a trace of a methylated uronic acid.

8. The fractions were present in the approximate proportions, 2:3-dimethyl methyl-L-fucosides 20%; 3-methyl methyl-L-fucosides 57%; methyl-L-fucosides 20%, suggesting that the basic unit in the structure of the methylated polysaccharide was 3-methyl L-fucose.

9. A tentative structure is suggested for the fucose-containing portion of the molecule.

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